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CEREBELLAR INFLUENCE ON PYRAMIDAL-TRACT NEURONES

BY K. L. CASEY AND A. L. TOWE

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(*Received* 23 *November* 1960)

Recent anatomical and electrophysiological investigations suggest that the anterior vermis of the cerebellum strongly influences the activity of the cerebral cortex. Moruzzi (1941) brought forth evidence that stimulation of this region facilitated or inhibited corticofugal discharge from the motor cortex of the cat, and Henneman, Cooke & Snider (1952) recorded evoked potentials from the surface of the pericruciate cortex of the cat following stimulation of the anterior vermis or the paramedian lobule. Whiteside & Snider (1953) showed that stimulation of the vermal cortex evokes potentials in a widespread region of the thalamus and upper brain stem; however, more restricted cerebellothalamic projections have been described by Combs (1959). Anatomical studies by Thomas, Kaufman, Sprague & Chambers (1956) and Cohen, Chambers & Sprague (1958) disclosed extensive vermal-fastigial projections to the thalamus and reticular formation via the uncinate fasciculus.

Because this cerebellocortical projection system involves the sensorimotor cortex (Henneman *et al.* 1952), stimulation of the anterior vermis should influence the activity of pyramidal-tract neurones. The present study was designed to elucidate the nature of this influence.

METHODS

Ten adult cats were lightly anaesthetized with chloralose (35–40 mg/kg) and paralysed with decamethonium bromide (1 mg/hr, intravenously). Respiration was sustained artificially through a tracheal cannula. The anterior lobe of the cerebellum, the pericruciate cortex, and the medullary pyramids were exposed. The calvarium on one side was removed, and the dura was reflected to expose about 2 mm^2 of tissue around the cruciate sulcus. The medullary pyramids were approached ventrally, through the basioccipital bone, by reflexion of the pharynx and the upper portion of the trachea. The cerebellar exposure, which accounted for a majority of the blood loss during the operation, was then effected by removing the calvarium immediately superior to the lambdoidal ridge and by removing enough of the bony tentorium to allow access to the dorsal surface of the anterior lobe. All exposed neural structures were protected from dehydration by a polyethylene film and liberal topical applications of warm NaCl 0·9 g/100 ml. Occasionally, intravenous 0·9%

saline was administered to compensate for some of the loss of blood and fluid during the operative and experimental procedures.

Bipolar needle electrodes were inserted into the central foot pad of the forelimb contralateral to the exposed cortex. Square-current pulses of 0·1 msec duration and 10 V amplitude were applied via an isolation transformer. The conditioning stimuli were applied to the midline culmen, centralis, or simplex lobules of the cerebellar cortex through silver wire bipolar leads separated 1 mm at the tips. The most consistent results were obtained by delivering to the cerebellar cortex a 40 msec train of 0·05 msec square-current pulses at a repetition rate of 312/sec; the duration of the conditioning trains used varied from 30 to 50 msec. The experimental results were not contaminated by the spread of cerebellar stimulating current to other structures, since a cerebellum which had been excised and soaked in 5 M-NaCl solution before being replaced could no longer affect the pyramidal tract discharge. Moreover, in intact preparations, direct stimulation of adjacent cerebellar cortex (lobulus simplex, folium and tuber vermis, and crus I or II) was totally ineffective.

The reflex pyramidal tract discharge following stimulation of the forepaw was recorded with a tungsten monopolar electrode inserted into the contralateral medullary pyramid (killed-end recording). A similar electrode simultaneously recorded the evoked primary response from the surface of the contralateral pericruciate cortex. The pyramidal and cortical responses were used to gauge the influence of cerebellar conditioning stimulation. Each response was fed to an oscilloscope through a Grass model P5 pre-amplifier; the over-all time constant of both channels was kept at about 150 msec.

RESULTS

Changes in the excitability of the pyramidal tract reflex

Stimulation of the forepaw consistently evoked a diphasic, positive-negative slow wave in the contralateral medullary pyramid. This response began 10–12 msec after forepaw stimulation and approximately 5 msec after the beginning of the evoked primary cortical response. Previous studies have identified this response as a reflex pyramidal tract discharge (Adrian & Moruzzi, 1939; Patton & Amassian, 1960). Prior cerebellar stimulation modified the latency and amplitude of the pyramidal tract response but did not alter its shape or duration. Amplitude was used as an index of the excitability of pyramidal tract neurones, as had been described previously (Zanchetti & Brookhart, 1955).

In the initial observations a variety of cerebellar stimulus parameters was tested. A 40 msec train of 0·05 msec pulses at 312/sec was most effective in producing consistent changes in the amplitude of the response, whereas single shocks, shorter trains, or lower frequencies were less effective. Longer trains, higher frequencies, or pulses of greater width did not alter the magnitude of the amplitude changes. Stimulation of the paramedian lobules, the tuber vermis, or crura I and II occasionally produced a small augmentation or attenuation of amplitude, but such changes were not consistent. On the other hand, stimulation of the culmen, centralis or simplex yielded marked and predictable amplitude changes. When the testing stimulus was applied in the first 5–10 msec after

the end of conditioning train (conditioning–testing intervals of from 0 to 5 or 10 msec) the peak-to-peak amplitude of the response was usually augmented. As the conditioning–testing interval was increased beyond 10 msec, a marked attenuation of the test response developed. This phenomenon is shown in Fig. 1.

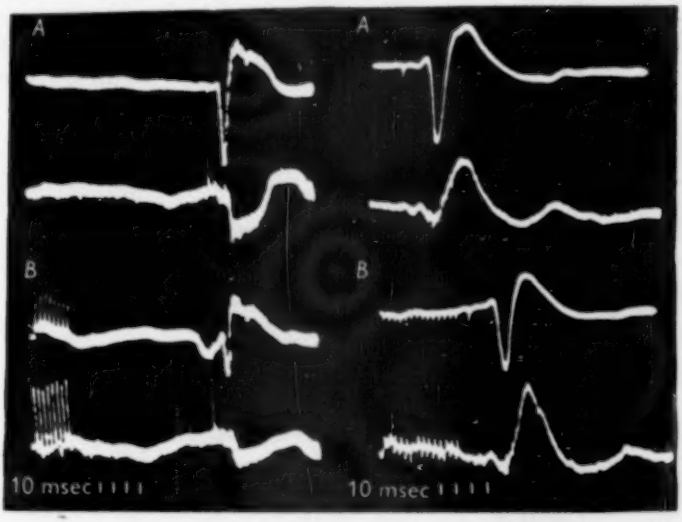

Fig. 1. Effect of cerebellar stimulation on reflex pyramidal tract response to cutaneous stimulation. *A*. Unconditioned responses. Top trace shows primary evoked response in postcruciate cortex; bottom trace shows reflex pyramidal tract response in medullary pyramid. *B*. Conditioned responses; top and bottom traces as in *A*. On left, C–T interval of 108 msec (measured from *end* of conditioning train). Positive phase of primary response is 60% of unconditioned amplitude; peak-to-peak amplitude of pyramidal reflex is 64% of unconditioned response amplitude. On right, C–T interval of 7 msec. Positive phase of primary response is 75% and pyramidal reflex is 120% of unconditioned response amplitude. Positivity at recording lead is down. Tracings on left were made after changing the sweep speed, the position of the recording electrodes, and after full penetration of the medullary pyramids (killed-end recording).

The time course of these amplitude changes was studied in ten cats. Conditioning–testing intervals of zero to 160 msec (measured from the end of the conditioning train) were used with six cats and conditioning–testing intervals of zero to 5000 msec were used with four cats. The mean amplitude of the unconditioned response was calculated from a series of 20–40 measurements made just before each conditioning–testing series. In this way, changes in the condition of the preparation were taken into con-

sideration. The mean amplitude of the conditioned response was similarly determined for a given conditioning–testing interval, and the effect of cerebellar conditioning was represented as the percentage of the unconditioned peak-to-peak amplitude. As is shown in Fig. 2*A*, an aug-

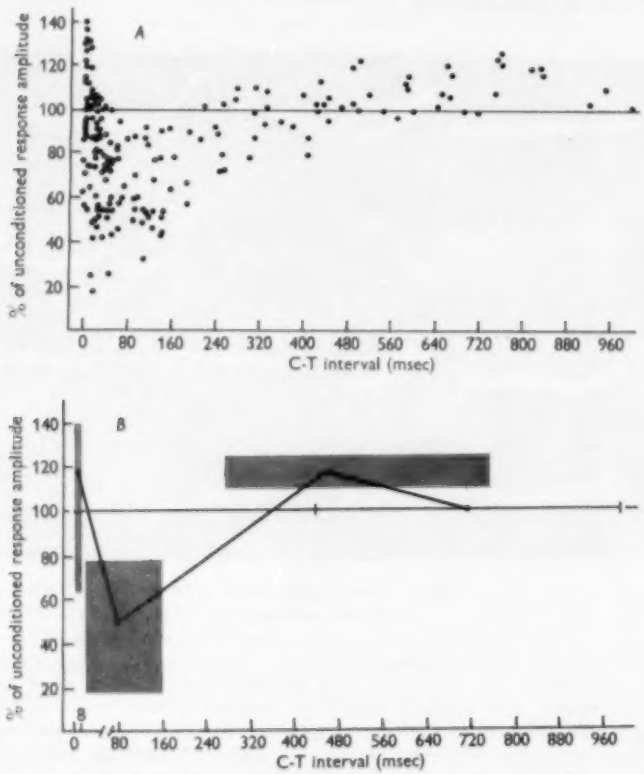

Fig. 2. Amplitude of pyramidal tract reflex at various times after cerebellar stimulation. *A*. Accumulated data from ten cats, showing general form and variability of interaction. *B*. Mean maximum changes in same ten cats, showing initial and final peak augmentations and intermediate peak depression. (For explanation see text.)

mented reflex pyramidal tract response was usually seen if the conditioning–testing interval was less than about 5 msec (35–55 msec after the beginning of the conditioning train). This initial phase of augmentation was difficult to demonstrate, for it began at some time during the conditioning train and was usually over shortly after the end of the conditioning train. Reliable measurement could not be made until the end of the conditioning train. None the less, the Wilcoxan test (ranked deviations) was significant

as a two-tailed test ($P = 0{\cdot}038$). The more appropriate randomization test (numbered deviations) was also significant as a one-tailed test ($P = 0{\cdot}027$) (Siegel, 1952). Both tests were applied to all observations made during the first 5 msec of the conditioning–testing interval; by a conditioning–testing interval of 10 msec the direction of the deviations was reversing. At greater intervals the response progressively decreased to about half its unconditioned amplitude and then recovered through a slightly augmented phase. No effect could be obtained at conditioning–testing intervals beyond 1000 msec.

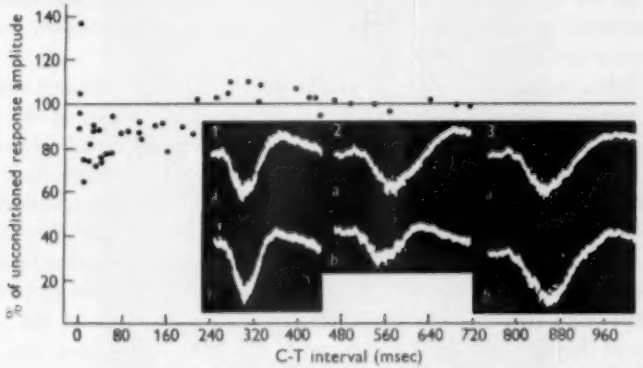

Fig. 3. Accumulated data from two of the cats entered in Fig. 2, showing minimal interaction effects. Inserted records: *a*, unconditioned pyramidal tract reflex; *b*, conditioned pyramidal tract reflex. 1, C–T interval zero, amplitude increased to 107 % of control amplitude. 2, C–T interval = 15 msec, amplitude decreased to 65 % of control. 3, C–T interval = 300 msec, amplitude increased to 117 % of control.

Two of the ten animals used in studying the time course of the interaction were different from the remaining eight in showing only minimal amplitude changes and a shorter time course of interaction. The data from these animals, shown in Fig. 3, should be compared with the data shown in Fig. 2*A*. Such a comparison reveals the three major variations observed: (1) the early augmentation phase was not present in all animals; (2) the magnitude of the response change varied greatly between animals; and (3) the time course of the change was also variable. Many of the individual variations observed probably represented differences in the physiological state of the preparation, especially of the exposed neural structures. No attempt was made to determine the effect of various anaesthetic agents or of different doses of chloralose.

The general pattern of the cerebellocortical interaction is revealed in Fig. 2*A*; the same data are presented in a different way in Fig. 2*B*. The maximal and minimal reflex discharge amplitudes were computed for each

animal and were found to occur within the ranges indicated by the hatched areas of Fig. 2*B*. The heavy line connects the mean maximum changes within each of the hatched areas and the mean point at which complete recovery occurred. The initial maximum augmentation was, on the average, 115% of the unconditioned reflex response amplitude, and occurred anywhere within the first 8 msec of interaction. The second, depressive, phase was the most striking; peak attenuation, which averaged 50% of the unconditioned amplitude, occurred at a conditioning–testing interval of 80 msec. The subsequent maximum augmentation averaged 117% of the control response amplitude, and occurred at 460 msec after the conditioning stimulus. In all preparations, unconditioned response levels returned within 440–1000 msec.

The latencies of both the unconditioned and the conditioned responses of the pyramidal tract were measured on enlarged images of the photographic records. The latency decreased in most conditioned responses in which *either* augmentation or attenuation of amplitude occurred. Latency changes could not be detected when the amplitude was unaffected by cerebellar conditioning stimulation. The mean latency of the unconditioned responses was $11{\cdot}40 \pm [0{\cdot}75]$ msec 0·06[150]; the mean latency of the conditioned responses with altered amplitude was $9{\cdot}86 \pm [2{\cdot}38]$ msec 0·19[150] (standard error is used). By t test the difference of the mean is significant beyond the 0·01% level ($P < 0{\cdot}001$); a marked non-homogeneity of variance is present.

Thus, stimulation of the anterior vermis of the cerebellum (1) changes the excitability of the pyramidal tract reflex in a complex but systematic way, and (2) decreases the latency of the reflex pyramidal tract response regardless of the direction of the amplitude change. The efficacy of the conditioning cerebellar stimulus depends upon the area stimulated and the parameters of stimulation and varies in magnitude and quality with the conditioning–testing interval.

Pyramidal tract discharge evoked by cerebellar stimulation

Stimulation along the anterior mid line of the cerebellum occasionally evoked either a purely positive or a diphasic, positive–negative slow wave which could be recorded from either medullary pyramid. The response could be evoked in six out of ten cats and there was considerable fluctuation in its appearance in any one animal. This response seemed to be quite sensitive to the amount of blood lost and to the manipulation of neural structures during operation. The discharge occurred about 20 msec after the beginning of a stimulus train or a single shock, and usually had an amplitude which was 30–40% of that of responses evoked by stimulation of the contralateral forepaw. Figure 4 shows records from an experiment

in which the pyramidal tract discharge was evoked by repetitive pulses at 312/sec (Fig. 4*A*, *B*) and by single 0·05 msec pulses (Fig. 4*C*, *D*). The responses to both types of stimulation were similar in amplitude, duration,

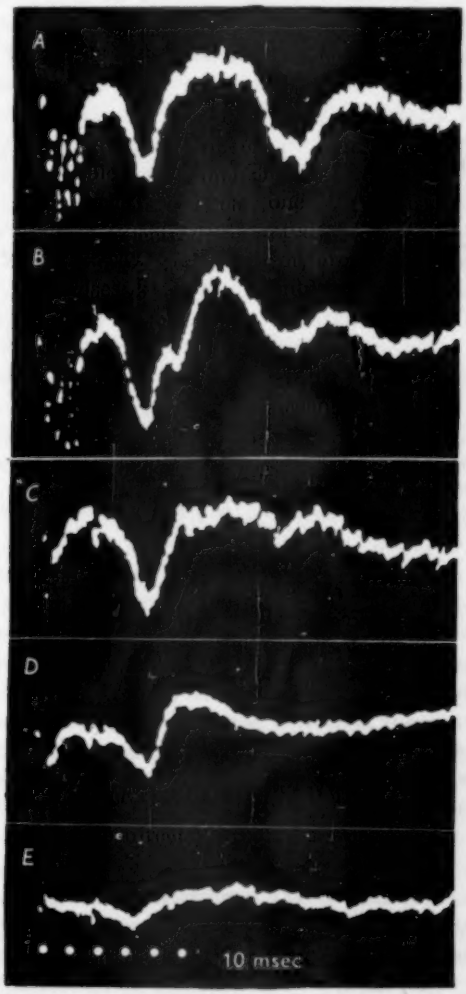

Fig. 4. Pyramidal tract discharge evoked by cerebellar stimulation. *A*, *B*: Repetitive stimulation at 312/sec; latency about 20 msec from beginning of stimulus train. Note later discharge in *A*. *C*, *D*: Single-shock stimulation; latency of about 20 msec. *E*. Response from same animal as in *D*, 2 min after ice had been placed on cerebellar cortex. Records *A*, *B*, *C* and *D* from four different animals.

and configuration. Repetitive shocks were often followed by an additional, even later, pyramidal tract discharge (Fig. 4*A*).

When ice was placed on either the cerebellar or the pericruciate cortex, the amplitude of the cerebellopyramidal tract discharge was considerably reduced within 2 min (Fig. 4*E*). The discharge returned to control amplitude 5–8 min after the ice was removed.

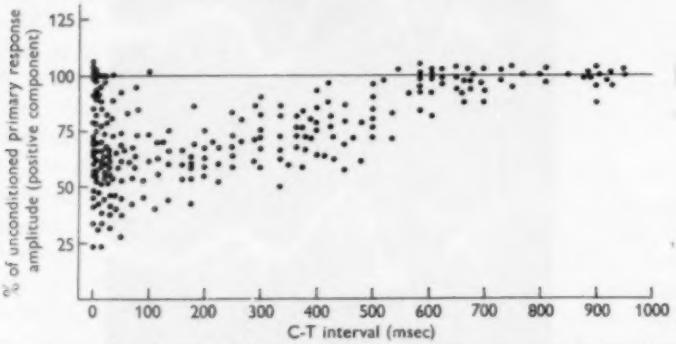

Fig. 5. Depression of positive phase of evoked primary response at various times following cerebellar stimulation; accumulated data from ten animals.

Changes in the amplitude of the positive phase of the evoked primary response

Stimulation of the anterior vermis reduced the size of the positive phase of the evoked primary response recorded from the pericruciate cortex. This phase was attenuated to approximately 65 % of its control value and continued to be depressed for 600 msec following the cerebellar stimulation (Fig. 5). No changes in latency could be detected and the negative phase was not systematically altered. The stimulus parameters were the same as those used for studying the cerebellar influence upon the reflex pyramidal tract discharge. Attenuation of the positive phase was independent of the amplitude of the simultaneously recorded pyramidal tract discharge (Fig. 1).

DISCUSSION

The experimental data clearly demonstrate the existence of a vermal-cortical system capable of influencing the activity of pyramidal tract neurones. Jansen & Brodal (1940) investigated the cortico-nuclear projections of the cerebellar cortex and found that the vermal cortex projects to the fastigial nuclei. The axons of the fastigial neurones form the uncinate fasciculus whose course has been described in detail by Rasmussen (1933). This fasciculus crosses the mid line within the cerebellum and then passes along the dorsal border of the brachium conjunctivum; a smaller,

uncrossed tract follows a similar course ipsilaterally. At the decussation of the brachium conjunctivum the fasciculus continues cephalad as an uncrossed projection into the mesencephalic tegmentum. The extent of the diencephalic continuation is not agreed upon by all investigators. McMasters & Russell (1958) describe a few fibres reaching the intralaminar complex of the thalamus while Thomas *et al.* (1956) and Cohen *et al.* (1958) report that many fibres reach the thalamus and project into the parafascicular, centromedian, ventral posteromedial, ventral medial, ventral lateral, lateral posterior, ventral posterolateral, ventral anterior, and other mid line and intralaminar nuclei of the thalamus. There is general agreement, however, that the uncinate fasciculi send a considerable number of fibres to the medullary, pontine and mesencephalic portions of the brain-stem reticular formation. Electrophysiological studies (Henneman *et al.* 1952; Gauthier, Mollica & Moruzzi, 1956; Snider, McCulloch & Magoun, 1949) furnish additional evidence for the existence of anterior vermal projections to the brain-stem reticular formation.

Since this cerebellocortical system appears to involve the reticular formation and, perhaps, many thalamic nuclei, one might expect rather complex effects on the activity of cortical neurones. Conditioning stimulation via a sensory (skin) nerve may either facilitate or suppress the antidromic activation of pyramidal tract neurones during conditioning–testing intervals of 10–25 msec (Li, 1959). Facilitatory effects on cortical sensory neurones and inhibitory effects on neurones in the motor cortex have been demonstrated following the stimulation of centromedian and ventralis lateralis nuclei, respectively (Li, 1956*a*, *b*), and both facilitatory and depressive alterations in the magnitude of the cortically-evoked pyramidal tract discharge follow repetitive stimulation of thalamic relay nuclei (Brookhart & Zanchetti, 1956). However, the excitability of pyramidal tract neurones was not influenced by stimulation of the mid line portion of the diffuse thalamic projection system, which represents the cephalic continuation of the reticular formation (French, 1960). The effect of stimulation of the brain-stem reticular formation on the excitability of the pyramidal tract discharge remains to be studied. Hagbarth & Kerr (1954) have demonstrated depression of peripherally evoked responses in the dorsal and ventral columns, mid-brain, cerebellum and sensory cortex during repetitive stimulation of the bulbar and mid-brain reticular formation or the ventromedial part of the anterior vermis, but this effect was abolished by the administration of pentobarbitone or chloralose. It will be appreciated, then, that stimulation of the anterior vermis may well activate several systems capable of influencing the excitability of the reflex pyramidal tract discharge. The present results represent the net effect of these influences.

Consideration of certain electrophysiological characteristics of the vermal-cortical system should yield some information relevant to its mode of operation. Single-shock stimulation of the anterior vermis evokes a surface-positive cortical wave after an interval of the order of 3·5–5·0 msec (Henneman *et al.* 1952) and causes discharges in some pyramidal tract neurones as much as 20 msec later. Since the excitability of the pyramidal tract reflex is usually increased for a short time following cerebellar stimulation (Fig. 2), the vermal-cortical system appears to exert a facilitatory influence which finally culminates in an excitation of pyramidal tract neurones. Repetitive cerebellar stimulation is capable of evoking successive pyramidal tract discharges (Fig. 3*A*); therefore, late excitatory cerebellar discharges could be evoked towards the end of the stimulating train. At conditioning–testing intervals of about 10 msec the test afferent volley is probably reaching the cortical population at some time close to the arrival of the latest possible excitatory cerebellar impulses. As Fig. 2 reveals, pyramidal tract reflex excitability is usually just entering the depressive phase at conditioning–testing intervals of about 10 msec. These facts furnish evidence that the excitatory phase of the vermal-cortical system is capable of initiating a blocking effect on the excitatory volley from the contralateral forepaw. Whether this blocking is inhibitory or occlusive at a cortical or subcortical level cannot be determined from the present studies.

It is rather surprising that those neurones which discharged during the depressive phase were also activated at significantly shorter latencies than those of the unconditioned population. The shorter latencies observed during the phases of increased excitability are to be expected on the basis of previous work (Morse & Towe, 1958; Towe & Kennedy, 1961). The latency shift during the depressive phase can be explained then, by assuming that an added excitatory input is still available to those pyramidal tract neurones not blocked by cerebellar discharge. Indeed, some excitatory input appears to persist throughout and beyond the period of depression, for as depression recedes a late facilitation appears. This late facilitation may represent a continuation of the effect of the early facilitatory system or it may reflect delayed facilitatory impulses arriving over different neural pathways. Prolonged effects might result from the activation of polysynaptic pathways at several points along the brain stem, within the thalamus, and within the cortex; in these experiments, the duration of cerebellar influence may be exaggerated by the repetitive cerebellar stimulation.

This vermal-cortical system, then, appears to operate via complex neural pathways which principally involve the reticular formation, the thalamus, and the pericruciate cortex. Activation of this system results

in a prolonged increase in the reflex excitability of some pyramidal tract neurones and a less prolonged depression of the reflex excitability of others; the onset of the depressive phase appears to be temporally related to the interaction of excitatory afferents from the anterior vermis and the contralateral forepaw.

The influence of cerebellar stimulation on the evoked primary response of the cortex is even more difficult to interpret. The origin of the evoked primary response of the cortex is unknown; the various views concerning its genesis have been reviewed by Towe (1956) and Woodbury & Towe (1958). Accordingly, discussion of the possible interpretations of the phenomenon described in this paper will not be attempted here. Two important observations should be emphasized, however: (1) the positive component of the primary response changes with cerebellar stimulation, while the negative component does not—i.e. the positive and negative phases do not behave as a unit—and (2) the amplitude of the positive component does not correlate with the excitability of pyramidal tract neurones.

SUMMARY

1. The effect of cerebellar conditioning stimulation on the excitability of the pyramidal tract reflex has been studied.

2. At conditioning–testing intervals up to 10 msec peripherally evoked pyramidal tract discharges were augmented by stimulation of the anterior vermis. There then followed a period of depression lasting up to about 400 msec and, finally, a late period of augmentation persisting up to about 1000 msec. The conditioning stimulus alone occasionally evoked a small pyramidal tract discharge after a latency of about 20 msec.

3. The latency of the conditioned pyramidal tract response was decreased regardless of the direction of the amplitude change.

4. The positive component of the evoked primary response of peri-cruciate cortex was depressed for 600 msec following the cerebellar stimulation; the negative component was not systematically altered.

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THE EFFECT OF SOME CATECHOLAMINES UPON A MONOSYNAPTIC REFLEX PATHWAY IN THE SPINAL CORD

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In 1937 Schweitzer & Wright described an inhibitory effect, following intravenous injections of adrenaline, upon the knee-jerk reflex in cats anaesthetized with chloralose. They found that the depression was not related to the blood-pressure changes produced, and that there was often an increase in the strength of the reflex muscle contraction preceding the depression. Later experiments by Bernhard & Skoglund (1953) and by Sigg, Ochs & Gerard (1955) indicated that either enhancement or inhibition of the reflex could be observed after systemic administrations of adrenaline, the effect depending in part upon the extent to which higher levels of the central nervous system were left intact to influence the spinal neurones, and also upon the depth of anaesthesia. Sigg *et al.* showed that inhibition was the only observed reaction to intravenous adrenaline in the deeply anaesthetized spinal animal. The effects obtained were interpreted by Schweitzer & Wright as indicating a direct action of adrenaline upon the spinal neurones, and this view has been accepted by later authors. On the other hand, in their studies of the effects of certain drugs on single motor neurones of the spinal cord Curtis, Eccles & Eccles (1957) failed to find any actions of adrenaline other than those which could be interpreted as due to vascular changes.

Interest in a possible direct effect of the catecholamines upon cord neurones has been raised again as an extension of attempts to identify an active ingredient in extracts of central nervous system (defined as containing Factor I) (Florey, 1954), which cause a reversible inhibition of the knee-jerk reflex following topical application to the exposed cord (Florey & McLennan, 1955) and seem to contain a substance active at inhibitory synapses (McLennan, 1960*b*). Factor I is able also to cause inhibition of impulse generation in the slowly-adapting neurone of the stretch receptor organs of crayfish (Florey, 1954), and in a recent study of a number of known compounds on this preparation McGeer, McGeer & McLennan (1961) showed that the catecholamines were active. Average threshold concentrations required to prevent impulse generation were: for L-

adrenaline, 18 μM/l., for DL-noradrenaline, 1·9 μM/l. and for 3-hydroxytyramine 0·1 μM/l. There was some indication that D-noradrenaline was considerably more active than the L-isomer. Since 3-hydroxytyramine is a normal constituent of the brain (see e.g. Carlsson, 1959) and would therefore be expected to occur in the Factor I extracts, and since it is by far the most active compound so far known to affect the crayfish neurone, these experiments were carried out to investigate its effects upon the knee-jerk reflex as possibly explaining the actions thereon of Factor I. The relationship of the results obtained to inhibition of the reflex by stimulation of the bulbar reticular formation (Magoun & Rhines, 1946) has also been explored.

METHODS

All experiments have been performed on cats weighing 2·8–3·2 kg, anaesthetized with chloralose (65 mg/kg) and urethane (250 mg/kg). Movements of the leg in response to stimulation of the patellar tendon by means of an automatic hammer (Schweitzer & Wright, 1937) were recorded on a kymograph, together with the carotid blood pressure in some experiments. Taps were delivered to the tendon at rates of 6/min or 12/min.

Substances were administered intravenously into the radial vein, or topically to the spinal cord. For the latter, the vertebral column overlying segments L 7–S 2 was opened from the dorsal side, the dura covering the cord removed, and the cord itself raised up on small glass rods to ensure that the applied solutions reached all parts of the surface. All solutions to be applied to the cord were warmed to 35° C, and between tests the cord was covered by a pool of warm sodium chloride solution, 0·9 g/100 ml.

The catecholamines used were all hydrochlorides, obtained commercially. Since there appears to be a contaminant in the 3-hydroxytyramine as supplied (McGeer *et al.* 1961) it was recrystallized from alcohol-ether before use. Solutions were made freshly in 0·9% sodium chloride solution.

Factor I extracts were prepared by the method of Florey & McLennan (1955). In some experiments a more purified material obtained by passage of the crude extract through a cellulose column, and recombination of the two active fractions resulting (McLennan, 1960*a*), was used, with identical results on the reflex activity.

Stimuli to the ipsilateral bulbar reticular formation were delivered with a bipolar concentric needle electrode placed in position after fixing the cat's head in a stereotaxic instrument. Points stimulated were in the area described by Magoun & Rhines (1946). In the majority of experiments the electrode tip lay within the limits of 12–14 mm posterior, 1–2 mm lateral and 7·5–9 mm below the Horsley–Clarke zero co-ordinates. Stimulation at a frequency of 50/sec, 0·1 msec duration and 3–5 V was sufficient to cause a marked inhibition of the reflex. At the end of each experiment the brain was removed and fixed in formalin and the position of the electrode determined after cutting sections of the tissue and staining with cresyl violet.

RESULTS

Intravenous administration

Schweitzer & Wright (1937) reported that the intravenous administration of 100–400 μg of adrenaline led in some cases to complete abolition of the knee-jerk reflex after a latency of less than 4 min, and the recovery from the depression was slow and sometimes incomplete. They noted also

that the inhibition was frequently preceded by a period of enhanced reflex contraction. Sigg *et al.* (1955) essentially confirmed these results, without, however, finding that complete abolition of the reflex was ever obtained; they further stated that noradrenaline gave similar but less pronounced effects.

The findings of Sigg *et al.* have been confirmed in the present series of experiments. The intravenous administration of 100–400 μg of L-adrenaline

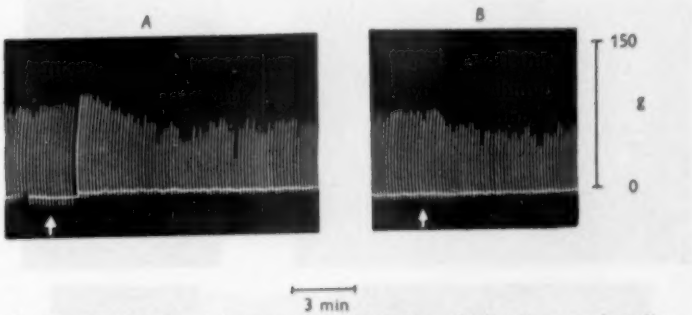

Fig. 1. The effects of the intravenous administration of (*A*) 150 μg L-adrenaline HCl and (*B*) 150 μg DL-noradrenaline HCl on the knee-jerk reflex.

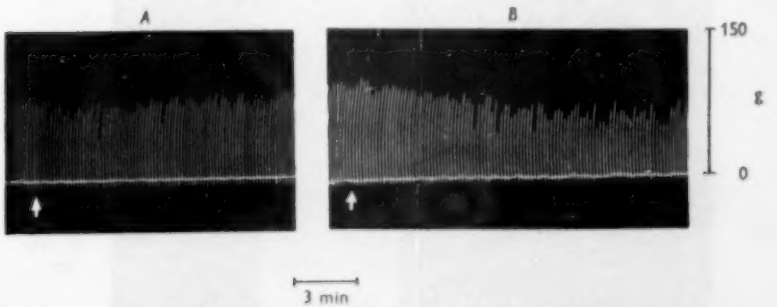

Fig. 2. The effect of the intravenous injection of 400 μg of 3-hydroxytyramine on the knee jerk, (*A*) before and (*B*) 10 min after the administration of β-phenylethylhydrazine hydrogen sulphate 10 mg/kg.

hydrochloride resulted normally in an enhancement of the reflex movement after a delay of 1–2 min, succeeded by depression of the reflex over the next 5–10 min with slow recovery thereafter (Fig. 1*A*). A similar but less marked effect was observed with equivalent amounts of DL-noradrenaline (Fig. 1*B*). Complete abolition of the reflex was never observed.

By contrast, 3-hydroxytyramine in equivalent dose was considerably less active than adrenaline in causing depression of the reflex and often had no

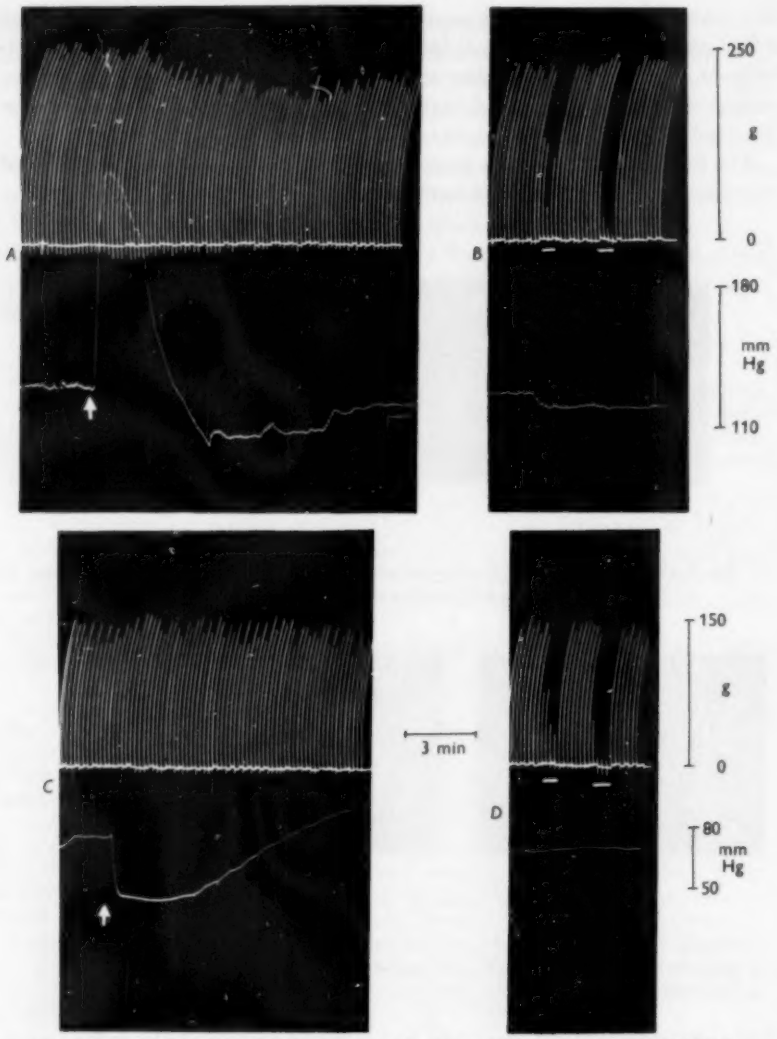

Fig. 3. The effect of Dibenzyline on the knee-jerk reflex and the carotid blood pressure in response to 300 μg adrenaline intravenously and on the inhibition produced by stimulation of the bulbar reticular formation. *A* and *B*, before; *C* and *D* 30 min after Dibenzyline, 15 mg/kg, I.V. Two strengths of reticular formation stimulation (2½ and 5 V) are shown in *B* and *D*.

detectable effect (Fig. 2*A*). That some activity in this respect was possessed by 3-hydroxytyramine was indicated, however, by the observation that after treatment of the animal with an inhibitor of monoamine

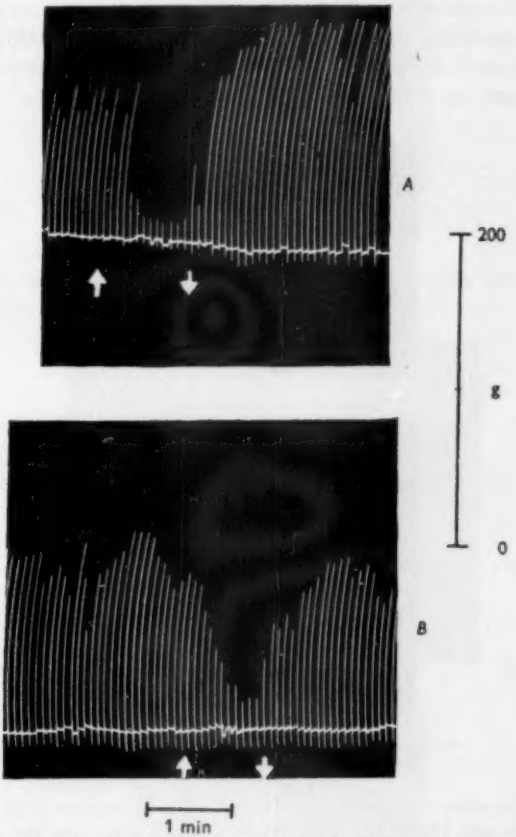

Fig. 4. The effect of topical application of Factor I solution to the exposed spinal cord on the knee-jerk reflex, (*A*) before and (*B*) 90 min after chlorpromazine, 15 mg/kg, I.V. The solution was applied to the cord at the first arrow in each case, and washed off with saline at the second. Note the post-inhibitory potentiation in (*A*).

oxidase (β-phenylethylhydrazine), an inhibition of the reflex appeared (Fig. 2*B*), and the pressor action of the 3-hydroxytyramine was prolonged. The depressant action of adrenaline on the reflex could similarly be potentiated by this agent.

The inhibition produced by adrenaline could be prevented by the administration of Dibenzyline (phenoxybenzamine), chlorpromazine or promazine. This is shown for Dibenzyline in Fig. 3. These drugs block excitatory adrenergic receptors ('alpha' in the terminology of Ahlquist (1948)). Dichloro*iso*proterenol (1-(3,4-dichlorophenyl)-2-*iso*-propylamino-ethanol hydrochloride), which specifically blocks the inhibitory 'beta' receptors (Powell & Slater, 1958) had no effect on the depression of the reflex produced by intravenous adrenaline.

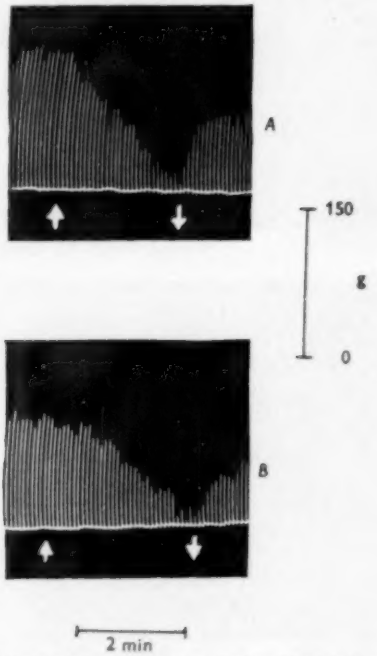

Fig. 5. The effect of topical application of 3-hydroxytyramine 50 g/l. to the cord, (*A*) before and (*B*) 30 min after Dibenzyline 20 mg/kg, I.V. The solution was applied at the first and washed off at the second arrow.

Topical administration

Florey & McLennan (1955) first reported the prompt and reversible inhibition of the knee-jerk reflex following topical application of concentrated solutions of Factor I to the exposed spinal cord. The effect is shown in Fig. 4*A*. A similar inhibition can be observed following the application of a drop of a solution of 3-hydroxytyramine to the cord (Fig. 5*A*). The concentrations of 3-hydroxytyramine which have been used in these

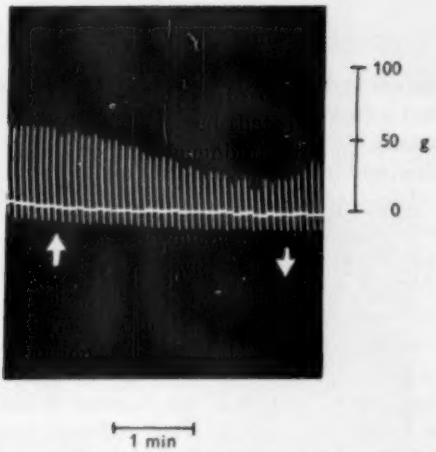

Fig. 6. The largest observed inhibition of the knee-jerk reflex obtained with DL-noradrenaline 100 g/l. applied to the cord.

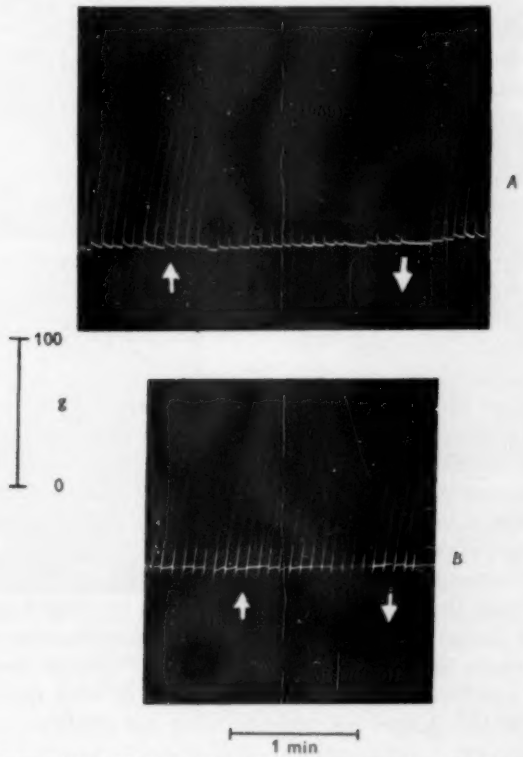

Fig. 7. Blocking of the inhibitory effect of 3-hydroxytyramine (50 g/l.) applied to the cord, by strychnine. (*A*) before and (*B*) after strychnine sulphate 0·1 mg/kg I.V.

experiments are high (25–50 g/l.), but inhibition can be obtained with less concentrated solutions provided that sufficient time of contact with the cord is allowed.

Adrenaline and noradrenaline are much less active than 3-hydroxytyramine when applied topically to the cord. Figure 6 shows the largest effect observed with 100 g/l. of DL-noradrenaline; 3-hydroxytyramine on the same preparation produced complete inhibition in about 30 sec with a solution of 25 g/l. Adrenaline was even less effective than noradrenaline.

The inhibitory effects of topically applied Factor I and 3-hydroxytyramine, unlike those of intravenous adrenaline, are not antagonized by the promazines or by Dibenzyline (Figs. 4 and 5). The effects of both are, however, prevented by strychnine in small doses (Fig. 7, and Florey & McLennan, 1955) and are similarly affected by the administration of dichloro*iso*proterenol, which blocks inhibitory 'beta' receptors, but has little effect on 'alpha' receptors (Powell & Slater, 1958) (Fig. 8). The effects obtained after topical and intravenous administration of the catecholamines are therefore different both in the relative strength of action of the three compounds tested and in the blocking of their effects by various pharmacological agents.

Stimulation of the reticular formation

Magoun & Rhines (1946) reported that stimulation of the ventromedial part of the reticular formation in the lower brain stem caused inhibition of spinal reflexes, a finding which has been amply confirmed. Recently, Cranmer, Brann & Bach (1959) reported that this reticular inhibition could be prevented by Dibenzyline infused intravenously at a rate of 0·025 mg/min, and restored by adrenaline infused or injected directly into the substance of the brain.

Evidence for a blocking action of a single large dose (20 mg/kg) of Dibenzyline (or of chlorpromazine) on the inhibitory effect of reticular formation stimulation could not be obtained in these experiments (Fig. 3). By contrast, the effect was antagonized by the administration of 7 mg/kg of dichloro*iso*proterenol (Fig. 9). The action of the adrenergic blocking agents on the inhibition due to reticular formation stimulation thus parallelled that obtained in the experiments in which 3-hydroxytyramine was applied to the cord, in that blocking agents for 'alpha' receptors were ineffective while that active at 'beta' receptors prevented the inhibitory effects. Strychnine in low doses was also able to antagonize partially the effect of reticular formation stimulation (Fig. 10): higher doses of strychnine could not be satisfactorily tested, since in such experiments the animals invariably jerked convulsively when the reticular formation was stimulated.

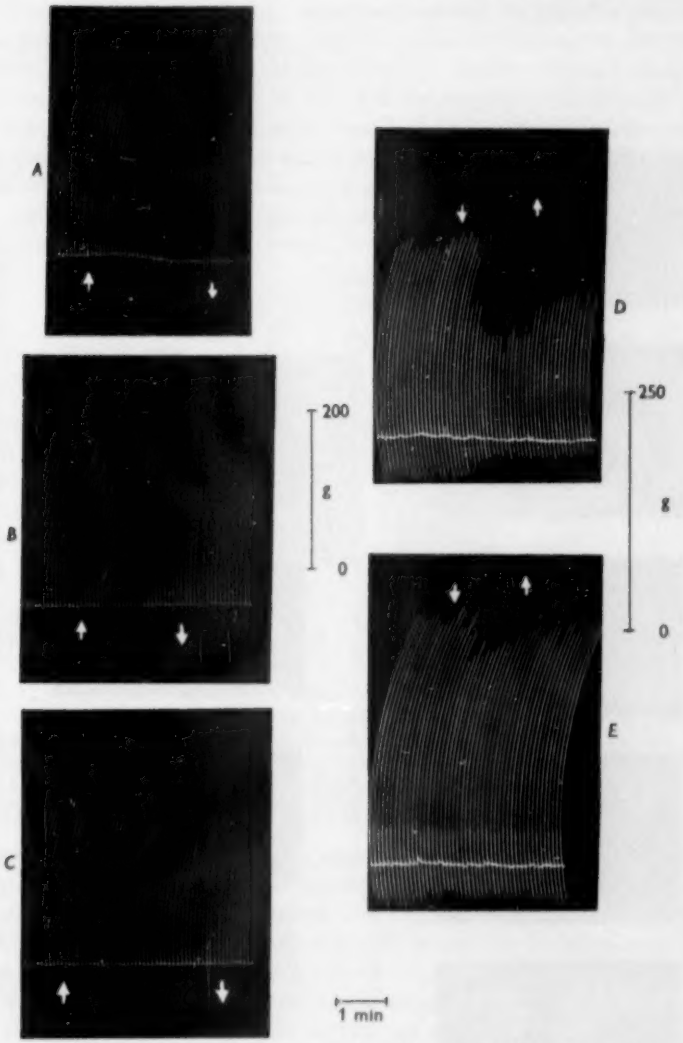

Fig. 8. The blocking action of dichloro*iso*proterenol on the inhibition of the knee-jerk reflex produced by topical application of Factor I (*A*, *B* and *C*) and, from a different experiment, of 3-hydroxytyramine, 50 g/l. (*D* and *E*). *A* and *D* before, *B* 1½ hr, *C* 3 hr, *E* 1¾ hr after dichloro*iso*proterenol, 10 mg/kg, I.V.

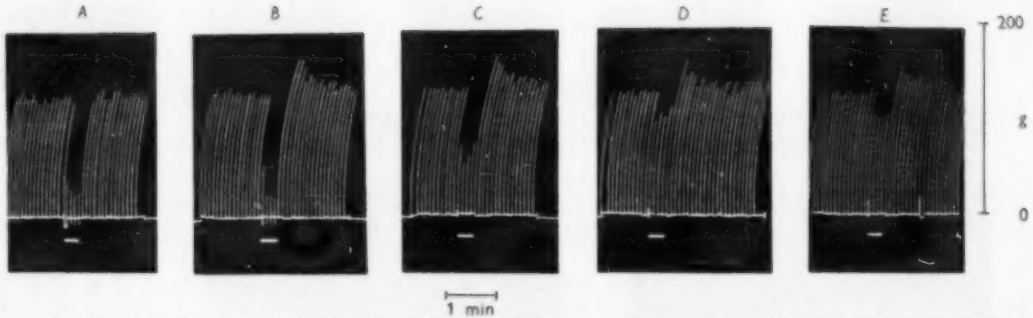

Fig. 9. Inhibition of knee-jerk reflex by stimulation (3½ V) of the reticular formation, (*A*) before, (*B*) 15 min, (*C*) 1 hr, (*D*) 1½ hr and *E* 3½ hr after dichloro*iso*proterenol, 7 mg/kg, I.V.

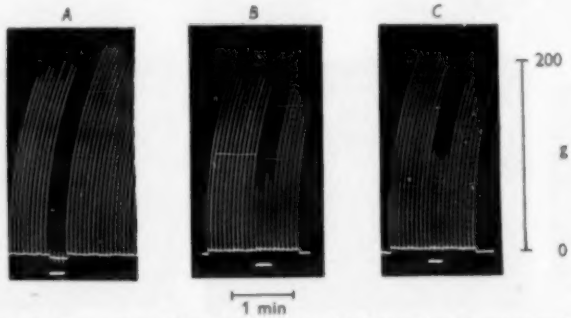

Fig. 10. Blocking action of strychnine on the inhibition due to reticular formation stimulation (5 V). (*A*) before, (*B*) after strychnine sulphate, I.V., 0·025 mg/kg and (*C*) after 0·05 mg/kg.

Treatment of an animal with reserpine depletes its brain of the catecholamines, including 3-hydroxytyramine (Carlsson, Rosengren, Bertler & Nilsson, 1957) and the level of the latter is rapidly restored after the administration of large doses of L-3, 4-dihydroxyphenylalanine (DOPA) (Carlsson, 1959). The experiments described above suggested the possibility that 3-hydroxytyramine was in some way implicated in the inhibition of the knee-jerk reflex brought about by reticular formation stimulation, and therefore that depletion of the endogenous stores of this substance might prevent the occurrence of the inhibition.

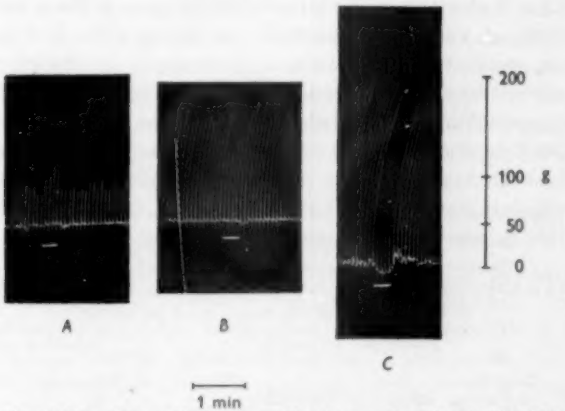

Fig. 11. Stimulation of the reticular formation in an animal treated for 3 days with reserpine. (*A*) control, (*B*) 2 hr after the administration of L-DOPA, 100 mg/kg, I.V. and (*C*) 3½ hr after DOPA. The strength of stimulation was 5 V in all cases. Note the small size of the reflex contractions in the reserpinized animal, and the enhancing effect of the stimulation; and the increased size of the contractions with reappearance of the normal inhibitory response after DOPA.

Cats were prepared by the intraperitoneal administration of reserpine 0·5 mg/kg for 3 days before the experiment, and a stimulating electrode was placed in the reticular formation at a point which in normal animals always gave a good inhibitory response (see Methods). In some cases the normal inhibitory response could not be obtained at all under these conditions (Fig. 11*A*), and instead stimulation usually gave rise to an enhancement of the reflex response. In other instances the usual inhibitory response was still produced by stimulation, but a much stronger stimulus than usual was required to elicit the effect. Following the intravenous administration of L-DOPA (100 mg/kg) the inhibitory response to the same stimulus gradually reappeared (Fig. 11, *B* and *C*). In those cases in which reserpine had caused the threshold for stimulation to be raised, it was reduced to the normal level following the administration of DOPA.

It was noted that in some experiments (as in that of Fig. 11) the size of the reflex contraction obtainable in animals treated with reserpine was small, and that this was greatly improved after the L-DOPA treatment. This observation suggests that 3-hydroxytyramine may, in addition to its inhibitory effects, have an excitatory function at some other site in the central nervous system; or since DOPA and 3-hydroxytyramine are precursors of noradrenaline and adrenaline, that the restoration of the stores of these substances is in some manner responsible for the increased response.

The finding that reticular formation stimulation in the animal treated with reserpine, at a site which normally would have resulted in inhibition of the reflex, can instead result in an enhancement would seem to indicate that this mechanism is not dependent on stores of catecholamines in the brain. Magoun & Rhines (1946) showed that stimulation of some points in the reticular formation resulted in increased reflex responses: the present results might be interpreted as indicating a generalized action in this direction which is normally masked by inhibition, but which appears when the inhibition is prevented by reserpine.

TABLE 1. The inhibitory effects of catecholamines and of Factor I on the knee-jerk reflex; and the actions of adrenergic blocking agents

	Inhibitory actions of				Inhibitory actions	
	3-hydroxy-tyramine	Nor-adrenaline	Adrenaline	Factor I	Antagonized by	Not antagonized by
Intravenous administration	0 or +	+ or ++	++	0	Dibenzyline, chlorpromazine	Dichloro-*iso*proterenol
Topical application	+++	+	0	+++	Dichloro-*iso*proterenol, strychnine	Dibenzyline, chlorpromazine
Reticular-formation stimulation	—	—	—	—	Dichloro-*iso*proterenol, strychnine, reserpine pre-treatment	Dibenzyline, chlorpromazine

0, no action; + weak action; ++ moderate action; +++ strong action.

DISCUSSION

A summary of the results described above is set forth in Table 1. It is apparent from the table that the actions of topically applied 3-hydroxytyramine and Factor I are similar, and that their inhibitory effect on the knee-jerk reflex is prevented by the same drugs as those which antagonize the inhibitory effect of stimulation of the bulbar reticular formation. A reasonable conclusion would seem to be that a catecholamine active at the 'beta' type of adrenergic receptors is in some way connected with the physiological inhibitory process. This statement is borne out by the

following points: (1) the inhibitory effect of stimulation of the bulbar reticular formation was prevented by treatment of the animal with reserpine, which depletes its stores of catecholamines; and was restored by the administration of DOPA, which replenishes the stores; (2) the action was also prevented by treatment of the animal with dichloro*iso*proterenol which specifically blocks the 'beta' receptors. The blocking of the effects of reticular formation stimulation by Dibenzyline, which was reported by Cranmer *et al.* (1959), was not obtained with the somewhat different conditions used in this study.

The results further suggest that the catecholamine involved is likely to be 3-hydroxytyramine rather than adrenaline or noradrenaline. This conclusion is reached since topical application of 3-hydroxytyramine to the exposed spinal cord caused a complete and reversible inhibition of the reflex, whereas noradrenaline had only a much weaker action in this respect and adrenaline little or none. This effect of 3-hydroxytyramine was prevented by administration of dichloro*iso*proterenol.

It seems likely that the inhibitory effect of intravenously administered adrenaline upon the reflex, originally reported by Schweitzer & Wright (1937), cannot be another aspect of the same mechanism as that described above. With topical application the order of potency (3-hydroxytyramine $\gg$ noradrenaline $>$ adrenaline) was the reverse of that seen with intravenous injection; however this might possibly be due to differences in penetration through the blood–brain barrier or to differing rates of destruction. The actions after intravenous administration, moreover, were abolished by Dibenzyline and by chlorpromazine, while these drugs were without effect on both the 'topical' inhibition and that due to reticular formation stimulation. Schweitzer & Wright showed that the effect was not related directly to vascular changes; but the interpretation of these results and of those obtained by Bernhard & Skoglund (1953) following intra-arterial administration to the cord is difficult.

The inhibitory action of extracts of mammalian brain (Factor I extracts) upon the knee-jerk reflex was reported by Florey & McLennan (1955). These extracts have properties in this regard very like those of 3-hydroxytyramine solutions, and the possibility implied by the findings of McGeer *et al.* (1961) on the crayfish stretch receptor neurone, that the extracts might owe part of their activity to 3-hydroxytyramine, is strengthened. The action of the pharmacological agents used here, i.e. the blocking of Factor I action by dichloro*iso*proterenol and by strychnine but not by Dibenzyline or chlorpromazine, parallelled that found for 3-hydroxytyramine and for reticular formation stimulation, and bears out the suggestion that Factor I extracts may owe their activity on the cord to 3-hydroxytyramine. It is noteworthy that some areas of the brain showing

high Factor I activity as assayed with the crayfish neurone, e.g. the caudate nucleus (Florey & Florey, 1958), also have high contents of 3-hydroxytyramine (Carlsson, 1959).

From the present experiments the suggestion that 3-hydroxytyramine is released from the endings of the reticulospinal tract neurones at their inhibitory synapses upon the motor neurones may be postulated. It should be noted that the inhibitory effects both of reticular formation stimulation and of topically applied 3-hydroxytyramine can be prevented by strychnine, which is known to inactivate inhibitory synapses at spinal motor neurones (Curtis, 1959).

Dr D. R. Curtis has kindly investigated the effects of 3-hydroxytyramine iontophoretically applied from a micro-electrode to single neurones of the spinal cord. The results which he has obtained were uniformly negative, and render the above suggestion of a transmitter action for 3-hydroxytyramine less attractive. The action of strychnine in blocking the effects of applied 3-hydroxytyramine, however, is difficult to explain in other terms in the light of present views on its mode of action.

SUMMARY

1. The inhibitory effects of adrenaline, noradrenaline and 3-hydroxytyramine on the knee-jerk reflex have been studied by intravenous administration or topical application to the exposed spinal cord, and compared with those produced by stimulation of the bulbar reticular formation.

2. Intravenous adrenaline caused a prolonged depression of the reflex, in confirmation of reports by other workers; noradrenaline was less potent in this respect and 3-hydroxytyramine almost inactive. The effects could be potentiated by administration of an inhibitor of monoamine oxidase, and that caused by adrenaline prevented by Dibenzyline or by chlorpromazine.

3. Topical application of 3-hydroxytyramine to the cord caused a complete and reversible inhibition of the reflex; noradrenaline was less active and adrenaline inactive. The effect of 3-hydroxytyramine was not blocked by Dibenzyline or chlorpromazine, but was prevented by dichloro-*iso*proterenol and by strychnine.

4. Stimulation of the bulbar reticular formation produced an inhibition of the reflex which was also unaffected by Dibenzyline or chlorpromazine but which was blocked by dichloro*iso*proterenol and by strychnine. Depletion of the catecholamines of the brain by pre-treatment of the animal with reserpine also prevented the effect of stimulation, and the inhibition could be restored by the administration of L-DOPA.

5. The inhibitory action of Factor I-containing extracts of mammalian brain on the reflex is similarly prevented by dichloro*iso*proterenol and strychnine, but is unaffected by Dibenzyline or chlorpromazine. It is suggested that Factor I extracts may owe their activity on the knee jerk to 3-hydroxytyramine.

6. The possibility is considered that the transmitter substance active at the inhibitory synapses of the reticulospinal tract upon motor neurones is 3-hydroxytyramine.

I am indebted to Dr George Ling and Dr P. L. McGeer for much helpful discussion and for generous supplies of the drugs used. Mr R. Walker rendered invaluable technical assistance. This work was supported by a grant from the National Research Council of Canada.

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SODIUM AND POTASSIUM MOVEMENTS IN THE UNSTRIATED MUSCLE OF THE GUINEA-PIG TAENIA COLI

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(*Received* 13 *March* 1961)

In 1949 Harris & Burn examined the movements of radioactive sodium and potassium in striated amphibian muscle, and concluded that the muscle behaved like a group of uniform cells surrounded by an extracellular space in which the ions moved by free diffusion. Creese (1954) found that the same hypothesis could be applied to striated mammalian muscle, but both workers were careful to point out that this formal interpretation did not prove that the sodium and potassium only occurred in one intracellular compartment. Harris (1953) found evidence that potassium in frog muscle exchanged at two different rates, and Weatherall (1959) observed a similar effect in rabbit atria. Waddell & Weatherall (1960) homogenized rabbit atria which had been exposed to radioactive potassium, and observed that the potassium in the intracellular particles exchanged more slowly than the remaining intracellular potassium, thereby demonstrating that heart muscle could not be regarded as a simple two-compartment system.

No comparable investigation of the kinetics of sodium and potassium exchange has been reported in unstriated muscle, although the electrophysiology of smooth muscle has been extensively studied. We have selected smooth muscle of the guinea-pig taenia coli for the present investigation because it is a thin piece of uniform tissue which has been examined by several techniques (see Bülbring, 1958, 1960 for references) and has already been found suitable for examination by radioactive tracer methods (Born & Bülbring, 1956). The experiments to be described here have been designed to answer two questions: where in the muscle are the sodium and potassium to be found, and according to what kinetic laws do they exchange with radioactive tracers? Only when these questions have been answered will it be possible to decide which fraction of the radioactive exchange corresponds to cation movements across the cell membrane, and hence to the electrophysiological behaviour of the muscle.

METHODS

Solutions

The dissection of the taenia coli was carried out in a solution which will be called 'cold dissection Ringer' and which was composed of (mM): Na^+ 144, K^+ 5·9, Ca^{2+} 3·7 and Cl^- 157.

The 'normal' solution used after the dissection period was a modified Krebs's saline prepared from isotonic stock solutions (Krebs & Henseleit 1932), containing (mM): Na^+ 137, K^+ 5·9, Ca^{2+} 2·5, Mg^{2+} 1·2, Cl^- 134, HCO_3^- 15·5, $H_2PO_4^-$ 1·2 and glucose 11·5, equilibrated with a gas mixture of 3 % CO_2 and 97 % O_2 to give a pH of 7·3 at 35° C. It differed mainly from the original Krebs's solution in a decreased bicarbonate, and an increased chloride content and in the absence of sulphate.

Dissection procedure

White guinea-pigs weighing about 500 g, of either sex, were stunned and bled out by the jugular veins and carotid arteries; the abdomen was opened and eight pieces of taenia were dissected with an *in situ* length of 25 mm. The fresh weight was determined and a loop of coloured cotton was next tied to each end of each piece while it was immersed in 'cold dissection Ringer'. A period of 1 hr was allowed for the whole procedure, so that many pieces of taenia coli from several guinea-pigs could be used in control experiments without the dissection conditions having to be altered.

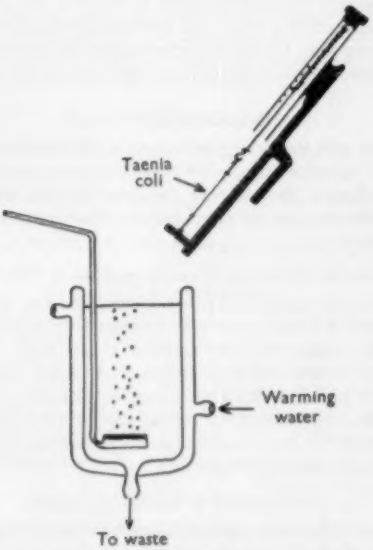

Fig. 1. Each taenia coli muscle was stretched on a calibrated spring balance which could be rapidly transferred from one organ bath to another during the course of an experiment.

At 1 hr the taeniae were set up on specially constructed 'spring balances' in 'normal' solution in an organ bath at 35° C, and the tension of each piece was adjusted to 2 g (Fig. 1). The pieces were then left for a further hour whilst the tension was readjusted if necessary to keep it at 2 g, and a pendular rhythm set in during this time in which the length and tension fluctuated by about ±5 %. After experiments lasting for various times the muscles were removed from the organ bath, the cotton was cut off and each piece was dried by holding one end of the taenia with a pair of forceps and wiping it across a sheet of black Perspex. The adhering solution was left as a trail, and the piece was considered to be dry when it no longer left this trail. It was weighed again in a small pot to give the wet weight, and the pot

was then placed in a drying oven at 105° C. After cooling in a desiccator the pots were re-weighed to give the dry weight (which was constant after 15 hr drying) and control pots were similarly treated to be used as counter-balances in the weighing.

Sodium and potassium analysis

1 ml. concentrated A.R. HNO_3 (sp. gr. 1·5) and 1 ml. concentrated A.R. $HClO_4$ (sp. gr. 1·7) were added to each pot, whose contents were evaporated to dryness at 130° C. After two further ashings the clear residue was dissolved in distilled water; since by this method the material was never removed from the original pot there were no losses due to transfer. Both sodium and potassium (and in some experiments lithium) were determined by flame photometry with a Beckman DU flame photometer, applying corrections for interfering light when necessary.

Preparations of radioactive solutions

The isotopes ^{24}Na and ^{42}K were supplied by the Atomic Energy Research Establishment, Harwell, where they were produced by neutron irradiation of 'Specpure' $NaHCO_3$ and K_2CO_3; these were contained in polythene jars to prevent any chemical contamination. The radioactive salt was dissolved in water and neutralized with hydrochloric acid to provide isotonic NaCl and KCl solutions. During the experiments with radioactive solutions, samples of the solution were taken at regular intervals so that the specific activity could be determined.

Counting procedure

The radioactive taenia and solutions were ashed in the normal way, but after the first ashing cycle they were counted with a G–M4 end-window counter, applying corrections for half-life, background and paralysis dead time. The half-life of the isotopes was checked from time to time in case there was any impurity, and the counting plateau and dead time were recalibrated when necessary.

Determination of the rate of uptake and loss of ^{24}Na and ^{42}K

In the uptake experiments pieces of taenia were immersed in 'normal' solution for 1 hr as usual, and at the end of this hour they were transferred to a bath containing normal but radioactive solution from which they were removed at intervals.

In order to determine sodium efflux other pieces of taenia were soaked in radioactive $^{24}NaCl$-'normal' solution for 24 hr. They were then transferred to an inactive solution and the rate of loss of ^{24}Na was determined in a similar way by removing the taenia at intervals.

The results are expressed as the relative activity (R) of the taenia, defined as (Specific activity of taenia)/(Specific activity of solution).

Determination of extracellular space

The extracellular space of guinea-pig taenia coli has been determined by chemical and histological methods, and the results obtained with conventional high-molecular-weight compounds (inulin, polyglucose) were compared with those using ions (Na^+, Li^+) and with those obtained from high-magnification electron micrographs of the muscle.

Inulin space

Inulin has been widely used by several workers for the determination of extracellular space, and our method of estimation has been based on that of Hubbard & Loomis (1942) for the determination of the substance in urine. Inulin 100 g was supplied by Thomas Kerfoots Bacteriological Sugars, and purified (Willaman, 1922), yielding a product from which the high and low molecular weight molecules should have been removed.

Preliminary experiments showed that the inulin content of a piece of taenia immersed in normal physiological saline solution which contained 2 mg inulin/ml. became constant after 20 min. Inulin spaces were determined after 2, 4 and 6 hr immersion, and controls were applied for the other solutes in the solution and for the reaction due to the taenia themselves.

Polyglucose space

A small sample of polyglucose was very kindly provided by Dr D. S. Parsons, Department of Biochemistry, University of Oxford, and was also used to determine the extracellular space. This substance was a large polysaccharide of uniform molecular weight which had been uniformly labelled with carbon-14. The polyglucose content of the taenia did not become steady for some hours after immersion in a solution containing this material, and observations were made after 2, 4, 6 and 8 hr. The taenia samples were afterwards homogenized and ^{14}C was counted in the homogenate with all precautions required for counting weak β-radiation.

Lithium space

The 'lithium space' was estimated after the initial dissection and equilibration periods, by changing the solution in the organ bath to one in which all the sodium had been replaced by lithium. (In doing this it was not possible to change from the normal solution to its exact lithium analogue, because all the necessary lithium salts could not be obtained sufficiently pure. The composition used in these experiments was therefore (mM): Na^+ (or Li^+) 147; K^+ 5·9; Mg^{2+} 1·2; Ca^{2+} 2·5; HCO_3^- 5·9; Cl^- 155; glucose 11·5; 99% O_2 + 1% CO_2). Pieces of taenia were removed from the bath at different times, and the lithium, sodium and potassium contents were determined by flame photometry.

Studies by electron microscopy

Electron micrographs of the guinea-pig taenia coli were taken at a magnification of 81,000 by Dr Shoenberg, Department of Biochemistry, University of Cambridge, who very kindly supplied us with a series of photographs. The pieces had been dissected and kept in 'normal' solution until fixation was started in 10% neutralized formalin. The extracellular space in each section was outlined on the photograph, and its area was measured with a planimeter.

V/A determinations

V/A is the ratio of cell volume to cell surface area. Pieces of taenia were dissected, fixed in 4% formalin Ringer's solution at different degrees of stretch, and embedded in gelatine to minimize shrinkage. Frozen sections were cut, stained with haematoxylin and eosin, and the stained sections were photographed. (We are indebted for these preparations to Miss Lucie Bülbring, Rotterdam.) In this way pictures were obtained of taenia coli muscles which had been fixed with lengths 50, 70, 96 and 125% of the *in vivo* length; and the diameter of about 100 cells in each section was measured by two independent observers.

Specific gravity

This was obtained by using a synthetic polymerization product of sucrose, 'Ficoll', which was kindly supplied by Pharmacia, Ltd., Copenhagen, Denmark. A series of 'normal' solutions was prepared with a specific gravity range from 1·03 to 1·07, but with constant tonicity, and the pieces of taenia were dropped into these to find a density in which they neither floated nor sank. Experiments were carried out with pieces of taenia which had been freshly dissected, and also with pieces which had equilibrated in the organ bath. Each piece of taenia was only used once.

RESULTS

Steady-state conditions

Results obtained with radioactive tracers may be most simply interpreted if it can be shown that the muscle is in a steady state, and that there is no net transport from one part of the system to another, but only

exchange. Preliminary experiments were therefore carried out to find if the isolated taenia coli behaved in this way, or if it changed appreciably during the course of an experiment.

Figure 2 shows the sodium and potassium content of whole taenia muscle at different times after the guinea-pigs were killed. The potassium content immediately after killing was 82 m-mole/kg fresh weight, but

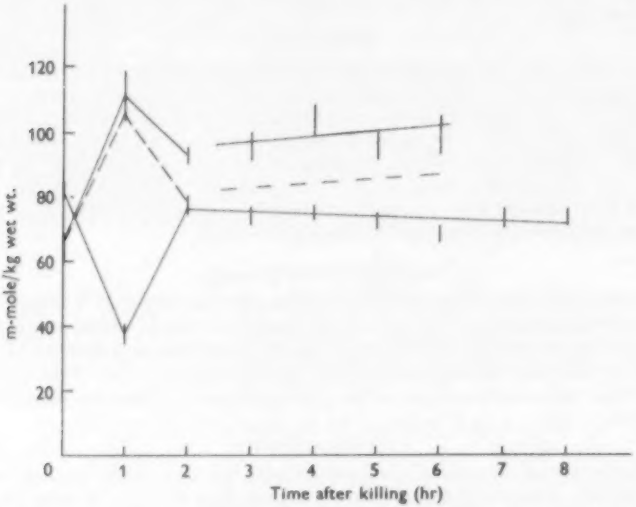

Fig. 2. The mean sodium (above) and potassium (below) content ($\pm 1 \times$ S.E.) of whole taenia coli muscles at different times after killing ten guinea-pigs. The first hour was the cold dissection period, and the changes at this time were partially reversed during the second hour, during which the taenia coli were being stretched in warm Ringer's solution. The best least-square regression lines are plotted for the results after the second hour. The interrupted line shows the sodium content after corrections had been applied for changes in (wet wt.):(fresh wt.) ratio and for the sodium which was initially adsorbed when the taenia was placed in dissection solution (see p. 438).

some potassium was lost during the first hour, which was the cold dissection period. However, the potassium content rose again when the pieces were placed in the warm oxygenated bath under tension, to reach a constant level (74 m-mole/kg weight) which was maintained for many hours. The results from 2 to 8 hr did not differ significantly from a straight line of zero gradient, and there was no significant variation from one animal to another.

In contrast, the sodium content immediately after the animals had been killed (65 m-mole/kg fresh weight) rose during the dissection period to

113 m-mole/kg wet weight, and fell slightly when the pieces were placed in the organ bath. The standard error of the sodium determinations was much greater than that of the potassium observations, which was to be expected, and an analysis of variance showed that there was significant variation from animal to animal but no significant change after different periods of immersion.

The ratio of the wet weight of a piece of taenia to its fresh weight when dissected was determined in 42 pieces which had been in the bath between 2 and 8 hr. This ratio was found to be $1{\cdot}02 \pm 0{\cdot}013$ and did not vary with the time in the organ bath, but was significantly lower than the ratio $1{\cdot}08 \pm 0{\cdot}014$ after the first hour's cold dissection. Table 1 shows the (dry wt.):(wet wt.) and (dry wt.):(fresh wt.) ratios of taenia muscle; in

TABLE 1. Changes in mean values of various properties during and after dissection. Standard errors of means are shown

Property	*In vivo* (10 determinations)	After 1 hr cold dissection (10 determinations)	2–8 hr after death (about 45 determinations)
Na content (m-mole/kg)	$65 \pm 4{\cdot}0$	$113 \pm 7{\cdot}2$	$97 \pm 2{\cdot}7$
K content (m-mole/kg)	$83 \pm 1{\cdot}5$	$38 \pm 2{\cdot}9$	$74 \pm 0{\cdot}8$
(Wet wt.)/(fresh wt.)	—	$1{\cdot}08 \pm 0{\cdot}014$	$1{\cdot}02 \pm 0{\cdot}013$
(Dry wt.)/(fresh wt.)	$0{\cdot}172 \pm 0{\cdot}007$	$0{\cdot}175 \pm 0{\cdot}008$	$0{\cdot}173 \pm 0{\cdot}004$
(Dry wt.)/(wet wt.)	—	$0{\cdot}160 \pm 0{\cdot}006$	$0{\cdot}168 \pm 0{\cdot}003$
Inulin space (ml./kg wet wt.)	—	—	333 ± 8

each case the graph of weight ratio against time was a straight line between 2 and 8 hr, whose gradient did not differ significantly from zero. The results of the inulin-space determinations also did not change significantly with time. It was therefore concluded from these preliminary experiments that the taenia came into a steady state after the initial trauma of dissection had passed, but that this steady state was not the same as its condition immediately after killing.

Control experiments

The biggest difference observed between taenia coli *in vivo* and *in vitro* was in the sodium content, which increased from 65 to a mean value of 97 m-mole/kg wet weight whilst the potassium content fell from 83 to 74 m-mole/kg wet weight. It was necessary to exclude artifacts or systematic errors in these results, by carrying out control experiments.

Taeniae coli from the same guinea-pig were dissected and put up in two different organ baths, one very poorly oxygenated whilst the gas was passed through the other as quickly as was possible. After 1 hr the pieces were taken down, ashed, and their sodium contents determined; in every experiment the sodium content of the well oxygenated taenia was slightly

greater than the sodium content of the poorly oxygenated, the mean difference being $5 \pm 3{\cdot}2$ m-mole/kg. Hence there was no indication that the sodium content of the taenia depended critically upon the degree of oxygenation.

It was also possible that irreversible changes took place in the tissue during the 1 hr cold dissection period. The standard dissection procedure was therefore compared with a quicker method in which the pieces of

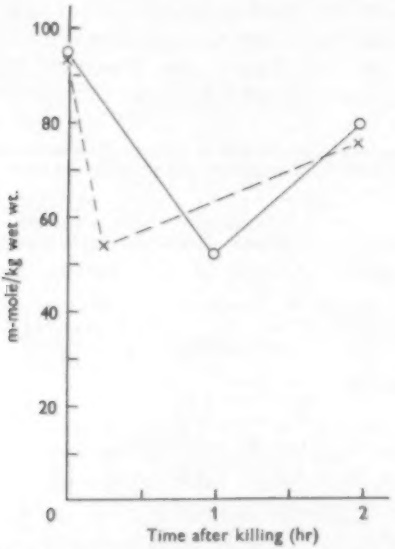

Fig. 3. Pieces of taenia coli dissected by the standard procedure (○), compared with other pieces from the same guinea-pig which had been more rapidly dissected in warm oxygenated modified Krebs's solution (×). In both cases potassium was lost during dissection and was restored to the muscle when it had been set up in the organ bath.

taenia were at no time allowed to leave warm oxygenated 'normal' solution. Figure 3 shows one of these control experiments in which potassium was estimated. The muscle lost potassium even when the rapid dissection method was used, and 2 hr after killing the restored value was very close to that in the pieces which had been normally dissected.

The standard drying procedure might have left a film of normal solution adhering to the muscle, which would have the effect of raising the sodium content and lowering the potassium. Pieces of taenia were therefore dissected, weighed fresh, dipped in 'normal' solution and reweighed after drying by the standard method. Seven out of eight lost weight slightly

when weighed the second time, the mean weight loss being $2{\cdot}3 \pm 0{\cdot}5\,\%$; but although the pieces had lost weight there had still been a significant ($P = 0{\cdot}001$) increase in the sodium content. This rose from a mean value *in vivo* of 63·6 m-mole/kg fresh wt. to 77·2 m-mole/kg wet wt., corresponding to a gain of 13·6 m-mole/kg; a most surprising observation after an immersion in normal solution which had been as brief as possible.

A length of cotton was attached to each piece of taenia when it was set up *in vitro*, but no such cotton was attached to the *in vivo* pieces. The weight of the cotton left on the taenia during the ashing procedure was therefore determined, and its sodium and potassium contents were determined and found to be very small.

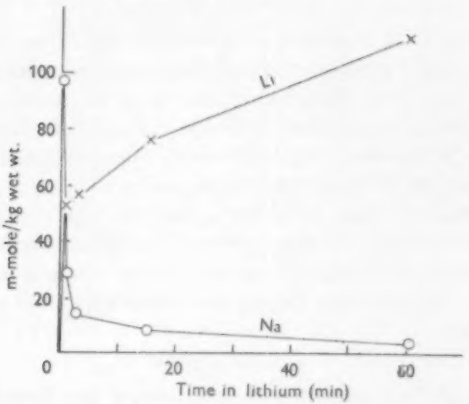

Fig. 4. An experiment in which pieces of taenia from the same guinea-pig were transferred to lithium solution. The rapid uptake of lithium at first was followed by a slower phase, and a small proportion of the muscle sodium remained in the tissue.

Extracellular space

The extracellular space was determined in the preliminary experiments using inulin, and was found to be $33 \pm 1\,\%$. However, when other methods were used in order to confirm this value, these did not all give the same result.

Figure 4 shows that the uptake of lithium by the taenia took place at two different rates; in the first minute there was a loss of sodium which was approximately equal to the gain of lithium and presumably represented extracellular exchange, but after this the lithium entered more slowly and there was always a small residual amount of sodium which remained in the muscle. In every experiment the rapid lithium uptake exceeded the quantity which would enter the inulin space, giving a mean lithium space of about 40 %.

When the rate of uptake was measured with ^{24}Na instead of Li, different results were obtained. All the sodium exchanged rapidly, so that a space calculated on the same assumptions would be 70%, a most improbable figure.

The rate of uptake of polyglucose by the taenia was very slow, and a period of 4 hr was required before a steady state was reached in which the muscle took up no more; even then the extracellular space measured with this very large molecule was only 22 ± 1 %; much smaller than the inulin or lithium space.

The measurements of the electron micrographs indicated an extracellular space of 20%.

Muscle size

The average weight of a piece of taenia coli was 17 mg, although pieces were dissected weighing between 4 and 26 mg in some experiments designed to investigate the effect of changing the size of the muscle. The length of all pieces was initially measured *in vivo*, and they were dissected in 25 mm lengths, although this measurement was only an approximate guide because the degree of contraction of the muscle was not constant. However, the length was measured again after the initial period of equilibration in the warm organ bath, when the muscle was subjected to a tension of 2 g. The mean length was only slightly greater under these conditions, $27{\cdot}2 \pm 0{\cdot}7$ mm, which suggests that the applied tension was not greatly different from that *in vivo*.

Muscle density

The specific gravity of pieces of fresh taenia was found to be 1·05 by flotation, and was not greatly altered if the pieces were dissected and equilibrated by our standard procedure.

Muscle shape

Histological sections of whole pieces of taenia coli were measured in order to determine the shape of the muscle, whilst other pieces were studied immediately after dissection and were measured with the graticule in a binocular dissecting microscope. The pieces had the shape of a rectangular block whose proportions were typically $0{\cdot}5 \times 1 \times 25$ mm; the average thickness was found to be 0·56 mm.

V/A

Each piece of taenia was made up of a bundle of smooth muscle cells arranged parallel to each other. The cell nucleus was found to occupy most of the thickness of the fibre; it was about one third to one half of the fibre length and a third of the fibre volume, but this was difficult to discern and cannot therefore be stated with certainty. When the muscle was stretched

the nuclei were stretched to almost the same extent (i.e. at 12·5 mm the mean length of the nucleus was 19·2 μ and at 37·5 mm it was 56·0 μ), whilst at the same time the diameter diminished. From measurements of large numbers of cells in preparations fixed at different lengths it was found that the transverse dimensions of the cells varied inversely with the length of the muscle. No allowance was made for the spindle shape, but a correction had to be applied for muscle shrinkage when the tissue was fixed; this was arbitrarily taken as a factor of 1·5.

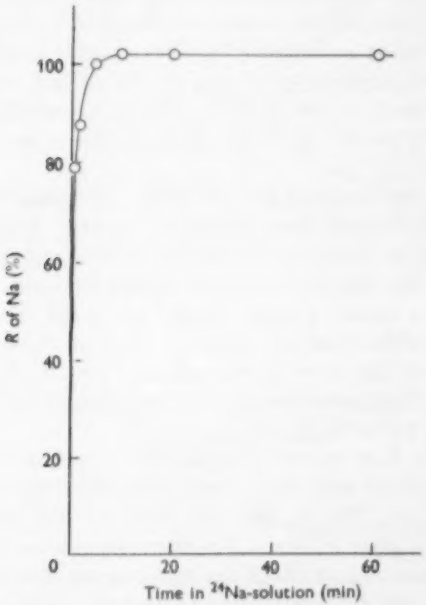

Fig. 5. Uptake of tracer sodium by six pieces of taenia coli muscle dissected from the same guinea-pig. Note that all the muscle sodium exchanged very rapidly.

A graph was constructed from which the cell diameter for any given experimental muscle length could be determined, and from which V/A was plotted on the same co-ordinates. The value of V/A which corresponded to our experimental conditions was 1·5 μ and was not critically dependent on the exact degree of muscle stretch.

Since the whole muscle was 0·5 mm thick, a central cell might be shielded from the bathing solution by a layer of 50 cells, representing a complex diffusion path for ions.

^{24}Na

The exchange of the sodium in the taenia with the ^{24}Na in the bathing fluid was very fast indeed, and after 5 min it was no longer possible to measure any difference between the specific activity of muscle and solution (Fig. 5). The useful period in which measurements could be made was therefore restricted to this short time. Furthermore, measurements could not be made too soon after the exposure to ^{24}Na began, because the procedure for drying a piece of muscle took 10–15 sec, and the exposure to ^{24}Na solution had to be long in relation to this time in order to avoid errors.

The initial half-time of ^{24}Na uptake was found to be 0·5 min, which was roughly equal to the rate of entry of sodium by simple diffusion processes alone (Fig. 7), and might suggest that all the sodium was extracellular. Indeed the kinetics of sodium uptake have been used in other tissues in order to determine the extracellular space, in the same way as we have used lithium.

Measurements were made of the rate of loss of sodium from radioactive taenia in order to extend these surprising results. After transfer for a period of 5 min to an inactive solution the specific activity was only 3% of its initial value, and the half-time of exchange was again initially 0·5 min. It was possible to detect a small slower exchange component by this method, and the relative activity was still 1% after 10 min in the inactive solution. However, the activity was already low by this time, and we could not follow this slow component for a longer period because the counting rate fell below background.

It was possible that the 24 hr exposure to radioactive solution had harmed the muscle in some way, and a simple control experiment was therefore carried out. The initial activity of the ^{24}Na solution had been 0·01 mc/ml. and a piece of taenia was therefore set up in a solution containing 0·15 mc/ml. and observed for many hours. During this time the tension was recorded and responses to acetylcholine and adrenaline were obtained at intervals, in a completely normal way. It was therefore concluded that no gross physiological damage had been done to the muscle.

^{42}K

The exchange of potassium was found to have a much slower time course than sodium, and even after 8 hr immersion the relative activity only approached 0·8 (Fig. 6) although it reached $0{\cdot}93 \pm 0{\cdot}03$ after 24 hr.

CALCULATION

We have found that the determinations of the extracellular space of the taenia coli have yielded varied, yet reasonable, results. The histological

estimate was the lowest (20 %) and the polyglucose space was only slightly larger; polyglucose is a very large molecule and can apparently only enter those most open intercellular spaces which are observed under the electron microscope. On the other hand the volume of distribution of inulin was appreciably larger (33 %), suggesting that this smaller molecule could penetrate spaces which were forbidden to polyglucose. Furthermore, the

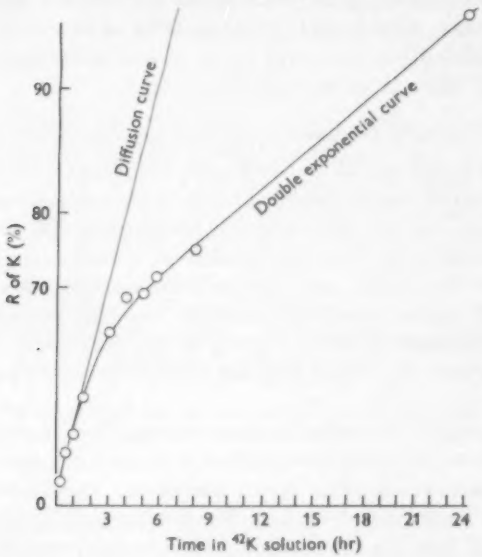

Fig. 6. Mean uptake of tracer potassium by pieces of taenia coli muscle dissected from twelve guinea-pigs. The divergence from linearity cannot be explained as a diffusion delay, but the results are well fitted by a double exponential equation.

inulin space remained constant from 2 to 6 hr, and preliminary experiments showed that the inulin could diffuse into or out of a taenia in 20 min; the polyglucose, however, required several hours to reach an equilibrium in the muscle. The lithium space of about 40 % was a little larger than that observed with inulin (which is not surprising with such a small mobile ion) and the lithium displaced sodium in the extracellular space with striking rapidity (Fig. 4). However, until the lithium method has been more thoroughly explored, we will take 350 ml./kg wet wt. as the extracellular space during the period 2–8 hr.

Extracellular ionic content

It is now possible to estimate the amount of muscle sodium and potassium which occur extracellularly in free solution, knowing the specific

gravity of the muscle to be 1·05. The volume of 1 kg of muscle is 952 ml., of which 350 ml. is extracellular. If this extracellular fluid is assumed to have the composition of 'normal' solution, it would contain a total of 2·1 m-mole/kg wet wt. of potassium and 48 m-mole/kg wet wt. of sodium, whilst any variation in the extracellular space would produce a proportionate change in these calculated quantities. Since the taenia contained 74 m-mole/kg of potassium and 97 m-mole/kg of sodium under our experimental conditions, there is still 71·9 m-mole/kg of K and 49 m-mole/kg of Na to be accounted for. This may occur in free intracellular solution, or may be bound inside or outside the cell.

Conclusions from isotonicity and electrical neutrality

There was a significant ($P < 0.001$) gain of sodium (13·6 m-mole/kg wet wt.) when pieces of freshly dissected taenia were momentarily dipped into normal solution, but the wet weight of the muscles did not change. This sodium which was taken up may therefore be bound to the tissue without contributing to its osmotic pressure, since the classical view (Peters, 1935) that the body fluids are always isotonic has been confirmed by more recent work (Robinson, 1960), leading to the conclusion that 'Osmotic equilibrium is probably never attained but always approached' (Manery, 1954).

It may be seen by inspection that no reasonable change of extracellular space and/or (wet wt.):(fresh wt.) ratio can explain the gain of sodium and loss of potassium which occur during dissection. Expressed in m-mole/kg wet wt., these ionic movements are almost equal and opposite (+48 of Na and −45 of K (see Fig. 2)), although the equality is destroyed when a correction is applied for the raised (wet wt.):(fresh wt.) ratio after dissection (+57 of Na and −42 of K). The muscle has on balance gained 15 m-mole/kg of cation, and it is a striking coincidence that this cation gain should be closely equal to the gain of sodium (13·6 m-mole/kg wet wt.) observed in the control experiments. The two pieces of evidence together suggest that the muscle rapidly took up about 15 m-mole Na/kg wet wt. when it was initially placed in physiological saline, and that this sodium remained at a site in (or on) the muscle. Such a site might exist in the collagen fibrils, and ions bound in this way could cause the apparent hyperosmolarity which has been reported in smooth muscle (Daniels, 1958). Furthermore, the rapidly exchanging sodium would not be entirely present in free extracellular solution, which explains in part why the extracellular space calculated from the uptake of sodium did not agree with the other observed values. A correction for this bound material may be applied to the results in Fig. 2 (interrupted line), and the gross uptake of sodium by the taenia is then almost equal and opposite to the loss of

potassium throughout the whole period of observation. There is still an increase in muscle sodium, and a corresponding loss of muscle potassium, but these ionic shifts do not deviate so much from the *in vivo* conditions.

During the period from 2 to 8 hr the (wet wt.):(fresh wt.) ratio remained close to 1 and the total [Na + K] in the muscle was 97 + 74 = 171 m-mole/kg. However an allowance may be made for the 13·6 m-mole/kg of sodium which initially entered and the content of unbound sodium and potassium would then be 171–14 = 157 m-mole/kg, being closer to the solution content of 150 mM.

Intracellular space

The sodium content (in m-mole/kg wet wt.) of the taenia was 97, of which 48 was extracellular and another 15 was probably bound, leaving 34 sodium and 72 potassium to be intracelluiar. The intracellular space was 952 − 350 = 602 ml./kg wet wt., but the cations did not necessarily have uniform access to the whole of this volume. Three alternative hypotheses may be considered:

(*a*) Taking the intracellular space as 602 ml./kg wet wt., the concentrations of sodium and potassium inside the cell may be calculated on the assumption (I), Table 2, that they are dissolved in this volume.

TABLE 2. The intracellular concentrations of Na and K calculated on two alternative assumptions (see text)

	AS I	AS II	
Extracellular space	350	350	ml./kg wet wt.
Density of muscle	1·05	1·05	g/ml.
Vol. of 1 kg of muscle	952	952	ml.
Dry wt. of 1 kg of muscle	168	168	g
Intracellular space	602	602	ml./kg wet wt.
Cytoplasmic space	602	401	ml./kg wet wt.
Extracellular volume (%)	36·8	36·8	none
Intracellular volume (%)	63·2	63·2	none
Total extracellular K	2·1	2·1	m-mole/kg wet wt.
Total K in muscle	74	74	m-mole/kg wet wt.
Total intracellular K	71·9	71·9	m-mole/kg wet wt.
Total cytoplasmic K	71·9	39·5	m-mole/kg wet wt.
[K] in cell	119	98	mM
Total extracellular Na	48	48	m-mole/kg wet wt.
Total Na in muscle	97	97	m-mole/kg wet wt.
Total residual Na	49	49	m-mole/kg wet wt.
Bound Na	15	15	m-mole/kg wet wt.
Total cytoplasmic Na	34	34	m-mole/kg wet wt.
[Na] in cell	56	85	mM
Cytoplasmic K + Na	175	183	mM
Ratio $[K]_i:[K]_o$	20	17	
Ratio $[Na]_i:[Na]_o$	0·4	0·6	

(*b*) Alternatively, it may be assumed that the ions are dissolved in the intracellular water. However, it is unlikely that the dry matter of the muscle has no sodium or potassium associated with it, and so this assumption will not be considered further.

(c) There may be more than one intracellular compartment, and the sodium and potassium may be distributed between these. Histologically the taenia has been shown to contain a large nucleus filling about one third of the cell, and it might be that this nucleus contained the potassium which exchanged slowly while the remaining potassium and the sodium were mainly in the cytoplasm (assumption II).

Potassium exchange

We have observed that the uptake of tracer potassium by the taenia coli took place at two different rates, and that it can be well represented by the double exponential equation (Fig. 6):

Relative activity $= 1-0{\cdot}45\exp(-0{\cdot}0013t)-0{\cdot}55(-0{\cdot}0144t)$, where t is the time in minutes from the start of exposure to ^{42}K. This type of behaviour cannot be explained on assumption (I) that there is only one intracellular compartment, unless the simple exchange in this compartment is modified in some manner:

(i) The slower exchange, which is first apparent after about 4 hr, might be due to the slow death of the tissue. However, it should be noted that the potassium content is maintained at a time when the slow phase of exchange is very pronounced.

(ii) A variation in the rate of exchange of different cells might explain the slower phase; thus Creese, Neil & Stephenson (1956) have suggested that a variation in cell size may cause the slower exchange which occurs in the rat diaphragm. The slow component of exchange is more pronounced in the taenia, however, and since we have no evidence of the variation between the rates of exchange of different cells in this tissue it is not possible to assess the importance of this effect.

(iii) A third explanation is that the first part of the muscle to exchange was the peripheral tissue, and that the slower exchange occurred in the central cells; tracer would have to traverse the extracellular space in order to reach the centre and diffusion delays in that space might slow down the availability of ^{42}K to the middle of the muscle.

In favour of this hypothesis it could be added that although the intracellular potassium concentration was high, diffusion of tracer had to take place through the extracellular space in which the concentration was much lower. However, a detailed investigation of the diffusion into a three-dimensional array of cells has indicated that this effect cannot explain the slow phase of potassium entry if any reasonable value be assigned to the diffusion coefficient; thus the diffusion curve drawn in Fig. 6 does not differ visibly from a straight line, although it has been calculated by

assuming the diffusion coefficient of potassium in the muscle to be only 10% of that in free solution.

In making this calculation it was not possible to use Harris & Burn's (1949) solution to the diffusion equations, because they had assumed that the exchange did not proceed to completion. We were, on the other hand, interested in the later and slower period when the exchange was nearly complete, and Dr B. Buck (Clarendon Laboratory, Oxford) has therefore solved the three-dimensional equations for the complete diffusion of tracer into a rectangular block of muscle. Neglecting any part played by rhythmic movement of the muscle and by intracellular diffusion delays these equations are:

$$\frac{D}{\lambda^2}\nabla^2 S^x = a\frac{\partial S^x}{\partial t} + b\frac{\partial S_1{}^x}{\partial t} \tag{1}$$

for the rate of accumulation of labelled ions in the muscle and

$$\frac{\partial S_1^x}{\partial t} = k_1 S^x - k_2 S_1^x \tag{2}$$

for tracer transfer across the cell membrane.

Constants
- S_o^x = concentration of tracer in the external solution.
- a, b, D and λ have the same meanings as in Harris & Burn's paper.
- l_1, l_2, l_3 are the linear dimensions of the muscle.
- k_1, k_2 are rate constants for the transmembrane exchange.

Variables
- x, y, z denote a point in the muscle.
- S_1^x = concentration of tracer in the intracellular fluid.
- S^x = concentration of tracer in the extracellular fluid.
- t = the time from introduction of tracer.

The appropriate boundary conditions are:

(i) For all *time* $S^x = S_o^x$; $x = 0, l_1$; $y = 0, l_2$; $z = 0, l_3$.

(ii) $t = 0$: $S^x = 0$; $0 < x < l_1$; $0 < y < l_2$; $0 < z < l_3$.

(iii) $t = 0$: $S_1^x = 0$; $0 < x < l_1$; $0 < y < l_2$; $0 < z < l_3$.

and it is important to note that S_1^x is not definable on the surfaces of the muscle; i.e. at the very edge there is no intracellular fluid.

The relative activity R of the whole muscle may then be found by calculating S_1^x and S^x, averaging over the whole muscle, and weighting the results in the proportions $a:b$ of extracellular to intracellular volumes:

$$R = aS_o^x F + b\frac{k_1}{k_2}S_o^x F' \tag{3}$$

in which F and F' are functions in space and time. This equation has been evaluated with the Oxford University Computing Department's Ferranti Mercury Computer, giving R to any desired accuracy, and the results were valid for long or short periods of immersion.

An alternative explanation based on assumption II is that the potassium occurs in two different compartments in the muscle, and in this case the observed double exponential relationship might well apply. The cells of the taenia contain a large nucleus (and other intracellular particles) and so readily satisfy the histological requirements for a series system in which the nucleus exchanges potassium with the cytoplasm and the cytoplasm exchanges with the extracellular space.

Sodium exchange

The exchange of sodium was much more rapid than that of potassium, and it seemed possible that the rate of entry of tracer sodium was limited

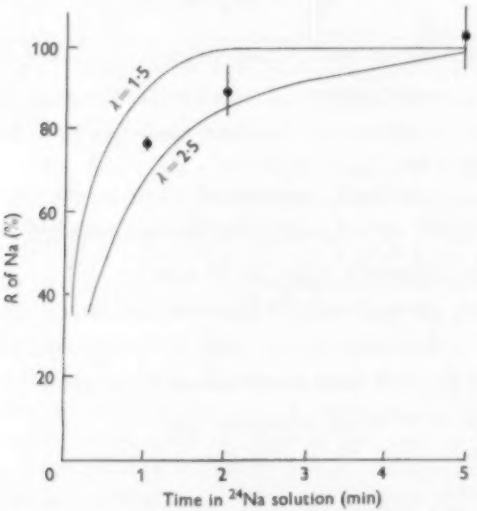

Fig. 7. Mean experimental uptake of tracer sodium by the taenia coli compared with the theoretical rate of diffusion into the muscle calculated for $\lambda = 1{\cdot}5$ and $2{\cdot}5$. It seems probable that the observed entry was limited by the rate of diffusion.

by the rate of diffusion into the extracellular space. Equation (3) has therefore been solved to calculate the rate of diffusion of tracer sodium into the taenia coli, and the results where two alternative values were assigned to the factor λ are shown in Fig. 7. We have no information concerning the value of λ which should be applied to the taenia, but it has been shown to

exceed 1·5 for skeletal muscle (Harris & Burn, 1949; Keynes, 1954; Creese, 1954). A value of 1·5 is not contrary to our experimental results, since the diffusive entry of tracer sodium would then be faster than the observed rate (Fig. 7), whereas if the value were as large as 2·5 the rate of entry by diffusion would be slower than the rate which we actually observed.

The rate of uptake of tracer sodium by the taenia is therefore consistent with an uptake determined by diffusive processes alone, and with λ between 1·5 and 2·5 being close to the value found in other muscles. In this case the observed rate would not measure the rate of transmembrane exchange, and the value of the rate constant calculated from these results would only be a lower limit.

Varying ion fluxes

It may be deduced that when radioactive material is being washed out of a muscle the graph of: $\log_e(a_1)$ plotted against time, in which a_1 = specific activity of the material in the cellular compartment should have a gradient of $-0{\cdot}693/T_{\frac{1}{2}}$, so that $T_{\frac{1}{2}}$ might be measured graphically in this simple way. However, the taenia was never in a steady state, but its activity was continuously varying. The electrical potential across the cell membrane was never constant, but rose and fell in slow irregular oscillations with a period of 1 or 2 min, and the efflux of potassium from a labelled muscle fluctuated in a similar way (Born & Bülbring, 1956). On top of these fluctuations was superimposed the rapid electrical activity of the action potentials, and the frequency of these also varied whilst sometimes they stopped altogether for short periods. It may be asked what the gradient $-0{\cdot}693/T_{\frac{1}{2}}$ really measured under these conditions?

In the lower part of Fig. 8 an ideally varying transmembrane flux m_0 is plotted against time. The equation of this flux is

$$m_0 = m_0' + m_0'' \sin 2\pi jt,$$

in which

m_0 = the size of flux at time t;

m_0' = the average value of m_0;

$2m_0''$ = the range over which m_0 varied; and

j = the frequency of the wave.

At Q the flux is large, the exchange constant k_2 is therefore also large and the instantaneous value of $-0{\cdot}693/T_{\frac{1}{2}}$ is numerically great. This is shown at Q' in the upper graph of Fig. 8, where $\log_e(a_1)$ has been plotted against t. The gradient at Q' is measured by the tangent to the curve, α, which is much steeper than the adjacent tangent β at R'. R' corresponds to R and to a period when the flux was small. It may be seen that a measurement of the gradient of $\log_e(a_1)$ instantaneously could give markedly different values of k_2.

For the ideal wave one may calculate how long observations must be made so that this variation cancels out. The results of Born & Bülbring (1956) indicate that there would be little error due to variations in the potassium ion flux, since the experiments on potassium uptake described in their paper lasted for several hours. On the other hand our sodium results

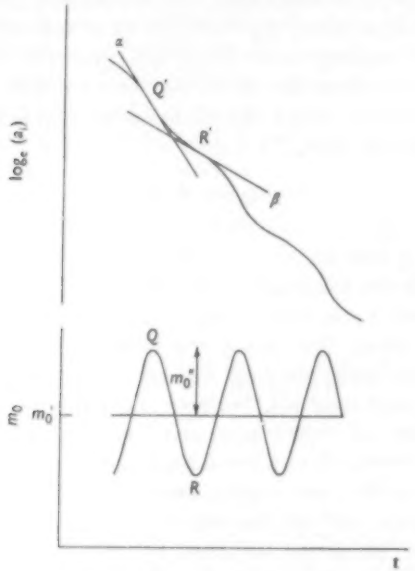

Fig. 8. Below. A varying flux m_0 across a cell membrane; the magnitude of this theoretical flux varies sinusoidally with time. Above. The specific activity in such a cell after it has been loaded with radioactive tracer and then placed in an inactive solution. This declines rhythmically because the transmembrane flux varies; such an effect has been observed for taenia coli muscle (Bülbring & Born, 1956, Fig. 2).

were obtained in less than 5 min, and should the instantaneous sodium flux vary to the same extent as the potassium flux it would be liable to variation from one experiment to another.

It is pertinent to ask what the average gradient of the graph $\log_e(a_i):t$ actually measured. In the case of the sinusoidal flux it may be shown by integration that this gradient represents the mean value of $-0{\cdot}69/T_{\frac{1}{2}}$ over one complete cycle; graphical integration confirms that this is also true when the flux varies in any irregular but cyclical fashion.

Ionic distribution

The cation distribution in the taenia has been calculated on two assumptions and the results are set out in Table 2. They will now be used

to determine the magnitude of the transmembrane sodium and potassium fluxes.

On assumption I the later slower potassium exchange is an artifact, and the initial rate constant for potassium exchange is therefore 0·0077 min^{-1}, applying to all the intracellular potassium. The rate constant for sodium exchange is at least 1·4 min^{-1}, and might be greater since the rate of exchange may be diffusion limited.

The transmembrane flux of ions may be calculated on the basis of the equation

$$\text{flux} = k_2(V/A)S_1 \quad \text{(Keynes \& Lewis, 1951)},$$

and we have found the ratio $V/A = 1{\cdot}5\ \mu$ in the taenia. This ratio is of course only approximate because an arbitrary factor has been used to assess the effect of muscle shrinkage during fixation, but it will presumably be the same for sodium and potassium. The intracellular potassium concentration is 119 mM, giving a transmembrane flux of approximately 2 pmole/cm^2.sec, and the intracellular sodium concentration is 56 mM giving 200 pmole/cm^2.sec on this assumption.

On assumption II we shall take the same value of V/A, although a correction might now be applied for the nuclear volume. The rate constant for potassium exchange is 0·01444 min^{-1}, since only 55 % of the potassium is involved, and the intracellular potassium concentration is 98 mM, giving a flux of approximately 4 pmole/cm^2.sec. The rate constant for sodium is unchanged, but the intracellular concentration is rather higher on this assumption (85 mM) and so the calculated flux is 300 pmole/cm^2.sec.

Transmembrane potentials

On assumption I the intracellular potassium concentration is 119 mM and the transmembrane concentration ratio is 20. This is not a very high value, and is still lower (17) on assumption II, agreeing well with the low membrane potentials which have been observed in the guinea-pig taenia coli by Holman (1958). The calculated potassium equilibrium potential is between $-60\log_{10}17 = -74$ mV and $-60\log_{10}20 = -78$ mV, while Holman's observations with micro-electrodes gave a mean resting potential of -51 mV with some values rising to -70 mV.

The sodium concentration within the cell membrane is 56–85 mM, corresponding to a sodium equilibrium potential of between $+25$ and $+14$ mV. Holman found a mean spike amplitude of 59 mV, giving a mean overshoot of $+8$ mV which approached the sodium equilibrium potential. The electrical behaviour of the cell membrane of the taenia might therefore be controlled by the movements of sodium and potassium ions alone although this does not rule out a contribution from other ions.

DISCUSSION

The movements of tracer sodium and potassium in unstriated muscle of the guinea-pig's taenia coli have been measured under conditions of constant load and therefore, probably, constant electrical activity. It has been shown that these movements did not follow simple exponential kinetics, and control experiments have been carried out in order to decide if the deviations may be explained as experimental artifacts, or if they are genuine properties of the tissue.

Faster dissection during which the muscle was always kept under warm oxygenated Krebs's solution did not change the ionic shifts which we observed *in vitro*, and the most powerful oxygenation of the solution was also without effect. In this the taenia behaved differently to the rat diaphragm (Creese, 1954) where the dissection procedure and oxygenation were critically important. The changes on dissection of the taenia appeared to be twofold; the rapid adsorption of sodium by the tissue when it was first placed into physiological saline, and the subsequent exchange of sodium for potassium. This exchange may be initiated by the stimulus of cutting the muscle, which is highly sensitive to mechanical stimuli.

A proportion of the muscle sodium behaved as if it was not osmotically active, and exchanged so fast that we have assumed it to be bound extracellularly. This bound sodium may in part explain the very rapid exchange with ^{24}Na which we have observed and the essential problem in the interpretation of the sodium movements is therefore to decide how much of the muscle sodium is truly intracellular and so taking part in transmembrane exchange. However, the flux of sodium ions was still very great even when full allowance had been made for such binding, and greatly exceeded the potassium flux. This was much slower, and took place at two different rates. One of these was very slow indeed and can contribute little to the transmembrane flux of potassium, but the faster component would correspond to about 2–4 pmole/cm^2.sec. The smooth muscle of the taenia coli therefore behaved rather differently from other muscles which have been studied, and we hope to investigate the differences further in order to establish if they are responsible for the peculiarities of this tissue.

SUMMARY

1. The movements of sodium and potassium in the smooth muscle of the guinea-pig taenia coli have been measured at 35° C in physiological saline solution. Under these conditions the muscle remained in a steady state for many hours, but the potassium content was lower and the sodium content correspondingly higher than it was immediately after killing a guinea-pig.

2. The potassium content of the muscle was 74 m-mole/kg wet wt.; the sodium content 97 m-mole/kg wet wt.; the extracellular space 350 ml./kg wet wt.; the (dry wt.):(wet wt.) ratio 0·168, and the ratio of cell volume to cell surface area 1·5 μ.

3. The exchange of muscle potassium was measured by means of radioactive tracers. 55 % exchanged with a half-time of 48 min and 45 % with one of 500 min.

Over 95 % of the muscle sodium exchanged with a half-time of 0·5 min, although a small fraction may have been exchanging more slowly.

4. When the sodium in the Ringer's solution was all replaced by lithium, there was a rapid loss of tissue sodium. However, some of the tissue sodium was retained under these conditions and exchanged very slowly.

5. The concentrations of sodium and potassium in the cell have been calculated as 56–85 and 119–98 mM respectively (Table 2).

6. The rate of movement of sodium in the tissue was limited by diffusion, but the rate of potassium movement was not so limited.

7. The flux of potassium across the cell membrane was 2–4 pmole/cm^2. sec, and that of sodium was many times greater.

We wish to thank Professor W. D. M. Paton, F.R.S., for the privilege of working in his Department, Dr Edith Bülbring, F.R.S. for much helpful discussion and advice on the properties of the taenia coli, and Dr E. M. Vaughan Williams for the loan of his spectrophotometer. P.J.G. is most grateful to the Royal Commission for the Exhibition of 1851 for a Senior Studentship, and K.H. to Pharmacia Ltd. for their support during this work.

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EXCHANGES OF WATER AND IONS BY KIDNEY SLICES DETERMINED BY A BALANCE METHOD

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Exchanges of water and ions between cells and their surroundings have often been deduced indirectly from the differences in composition between fragments of tissue which had been treated in different ways. This required the assumption that the differences in composition between two separate pieces of tissue accurately reflected the changes which would have occurred if a single piece of tissue had been transferred from one set of conditions to the other. How far this assumption may be valid depends, among other things, upon the basis chosen to express the results of analysis. When experimental conditions lead to gross swelling, figures expressed per unit mass of moist tissue are admitted to be misleading, but it has been claimed that results expressed in terms of unit mass of dry matter (usually referred to as dry 'weight') are satisfactory (Leaf, 1956). The argument from differences between the compositions of fragments of tissue to movements of ions into and out of cells still rests, however, upon the tacit assumption that a given mass of dry matter always represents the same number of cells. This assumption is perhaps most likely to be false for comparisons such as were made by Leaf (1956) between freshly cut tissue and slices which had been incubated under specified conditions; for an appreciable amount of material is lost from the tissue during incubation. An initial loss of more than 10% of the nitrogen of the tissue occurs during the earliest stages of incubation in manometric flasks, and although this initial loss may be chiefly of erythrocytes and debris from cells damaged during slicing, further and greater losses occur as incubation continues (Abei, 1952; Robinson, 1949). In proportion as the cells of incubated slices lose protein during an experiment, each kilogram of dry matter remaining will correspond to a greater number of cells than 1 kg of dry matter in the tissue used at the outset. If this change is ignored, the quantities of water and electrolytes found by analysis to be associated with 1 kg of dry matter at the end of an experiment will be assigned to a smaller number of cells than actually contained them. Any uptake of sodium, chloride and water by the cells will consequently be exaggerated, whilst losses of cellular potassium will be underestimated.

An attempt has therefore been made to apply a metabolic-balance technique to individual kidney slices, and so to deduce exchanges of ions and of water between the cells and their surroundings from changes in the amount and composition of the medium. Tissue slices are usually incubated in large volumes of medium, in order that only minimal changes in the composition of the medium may occur. In the present experiments small volumes of medium, comparable with the volumes of the slices, were employed, in order that exchanges with the tissue should lead to measurable alterations in the volume and composition of the medium. This has made it possible to observe exchanges between a particular fragment of tissue and its surroundings directly. The present paper is concerned with the effect of suppressing metabolic activity by chilling, and the results obtained by the new method have been compared with those deduced from analyses of tissue fragments.

METHODS

Medium. The 'ordinary medium' described earlier (Robinson, 1956), was used. It contained (m-equiv/l.): Na^+ 145; K^+ 5; Ca^{2+} 5; Mg^{2+} 2; Cl^- 140; SO_4^{2-} 2; and was buffered with phosphate (8 m-equiv/l.) at pH 7·4. Glucose (1 mg/ml.) was added before use.

Procedure. Slices were cut from the renal cortex of adult male hooded rats by Cohen's (1945) modification of the method of Deutsch (1936). The slices were usually put directly into about 50 ml. of medium maintained at 38° C and vigorously stirred by a magnetic stirrer. Oxygen was bubbled through the medium continuously. This preliminary treatment was intended to allow the slices to reach a reasonably uniform 'initial' condition of metabolism and composition, and also to remove tubular urine, erythrocytes and cellular debris. Microscopic (phase-contrast) examination of the sediment remaining after the slices had been removed showed a preponderance of erythrocytes.

After 20–30 min agitation in the warm oxygenated medium, sample slices were removed, gently blotted on filter paper, and transferred to small, weighed Pyrex tubes for drying and analysis. Similar slices were blotted and then put into small weighed, stoppered tubes, each of which contained about 0·1 ml. of medium. The amounts of tissue and of medium were determined by weighing the tubes before and after each addition. The tubes were shaken to bring the slices down into the medium, and then placed in crushed ice in the refrigerator, where they remained for approximately 1 hr with occasional shaking.

After this 'incubation', or immersion, in the cold, each slice was removed gently from its tube with forceps, together with as little medium as possible, and placed upon a small weighed piece of filter paper to remove adhering medium. The slice was promptly transferred to a weighed tube for drying and analysis, and the filter paper quickly reweighed to determine the amount of medium which had been carried away with the slice.

A small amount of debris remained in each incubation tube after the slice had been removed. The tubes were centrifuged to consolidate the sediment, and the medium carefully transferred by means of a small Pasteur pipette to 10 ml. stoppered Pyrex tubes for analysis. The amount of medium thus transferred was determined by weighing each tube before and after the transfer. The latter weighing also gave the amount of debris left in the tube. Despite the preliminary treatment of the slices, which might have been expected to remove all readily detachable debris, the amounts which remained at this stage averaged about 10 % (S.D. ± 3 % in 18 expts.) of the weights of tissue which had been placed in the tubes at the beginning of the experiments. On account of its greater water content, however, this debris contained only about 4 % of the dry matter of the original tissue. Phase-contrast

microscopy showed mainly cellular debris, with few erythrocytes present. 1 ml. of 0·1 N-HNO_3 was added to the debris in each tube after its weight had been recorded.

Analytical methods. The slices were dried overnight in an oven at 105° C and reweighed to determine their water content. The dried residues were extracted with 0·1 N-HNO_3 at room temperature and made up to a final volume of 10 ml. in stoppered Pyrex tubes, as described by Whittam (1955). In preliminary experiments half the slices from a single pair of kidneys were placed separately in 0·1 N-HNO_3 moist, and the other half were extracted with 0·1 N-HNO_3 after drying. These experiments revealed no significant difference between the amounts of Na and K recovered per unit weight of moist tissue. Hence previous drying did not seem to impair the extraction of Na and K from the slices, and it was feasible to determine water and ions on the same piece of tissue. 0·1 N-HNO_3 was added to the samples of medium recovered from the incubating tubes, to make in each case a final volume of 10 ml. for each 100 mg of medium. As the individual amounts of debris were small, the extracts from three incubating tubes were pooled and made up to 10 ml. with 0·1 N-HNO_3.

Sodium and potassium were determined in the acid extracts by means of an EEL flame photometer, using external standards made up in 0·1 N-HNO_3. There was no evidence of interference between sodium and potassium in the concentrations encountered.

Chloride was determined by titrating portions of the acid extracts with 0·001 N-$AgNO_3$ by the method of Cunningham, Kirk & Brooks (1941), as described by Hawk, Oser & Summerson (1954), using a Pye 'Scalamp' double-reflexion galvanometer, Number 7904/T.

RESULTS

Initial composition of slices as used

Nine sets of sample slices from the warm, oxygenated medium contained 74·9 % of water (S.D. ± 0·8 %) and had the average ionic composition shown in Table 1.

TABLE 1. Composition of tissue as used

(Averages and standard deviations from analysis of 9 samples)

	m-equiv/kg tissue	m-equiv/kg dry matter	m-equiv/kg water
Sodium	92 ± 3	366 ± 20	123 ± 4
Potassium	55 ± 4	218 ± 10	73 ± 6
Chloride	72 ± 14	291 ± 63	97 ± 19
$(Na^+)+(K^+)$			196 ± 8
$(Na^+)+(K^+)+(Cl^-)$			293 ± 15

Uptake of fluid by chilled slices

Changes in weight of slices and of medium. The initial weights of tissue used in 18 experiments ranged from 22·4 to 71·9 mg. In order to derive averages, the changes in weight were expressed in mg/100 mg of initial weight. All slices removed after immersion in the medium for 1 hr at 0° C were heavier than when they had been placed in the tubes. The average gain in weight was 13·5 mg/100 mg of initial weight (S.D. ± 4 mg). This increase in weight underestimates the swelling which had occurred. The slices removed from the tubes did not contain the whole of the swollen tissue, because the swollen debris left behind had also been part of the

tissue placed in the tubes initially. The amount of debris averaged 9·5 mg/100 mg of initial tissue (S.D. ± 3 mg). Hence the total increase in weight due to swelling was 23 mg/100 mg of initial tissue (S.D. ± 5 mg).

Actual losses in weight of medium in the same 18 experiments ranged from 6·4 to 18·1 mg. The average loss per 100 mg initial weight of tissue was 24 mg (S.D. ± 5 mg), and thus did not differ appreciably from the gain in weight of the slices.

The water content of the swollen slices. To test the validity of the method, the measured water content of slices removed from the tubes after chilling was compared with the water content calculated from the amount of water which had entered the slices from the medium. It was necessary to allow for the fact that some of the fluid which disappeared from the medium remained in the debris and so did not contribute to increasing the water content of the slices removed from the tubes. Since a few samples of debris which were dried contained about 90 % of water by weight, the amount of fluid taken up by each slice was estimated by subtracting nine-tenths of the weight of debris left in each tube from the loss in weight of the medium. It was further assumed that each slice initially had the same water content (74·9 %) as the similar slices which had been dried and analysed, and that it had lost dry matter amounting to one-tenth of the weight of debris. The expected final water content, calculated from the initial weight and water content, the weight of fluid gained, and the weight of dry matter lost, averaged 78·2 % (S.D. ± 0·7 %). This agreed well with the average of 78·4 % (S.D. ± 1·5 %) found by drying the swollen slices from the same 18 experiments.

The increase of a little over 3 % from the initial water content of 74·9 % is an insensitive index of the amount of water taken up by the tissue. Thus, if 100 mg of tissue containing 75 % of water took up 15 mg of water, it would finally contain 90 mg of water in a total weight of 115 mg, so that its water content would become 78·3 %. The uptake of 15 % of its weight would increase the water content of the tissue by a little over 3 %.

The external balance of electrolytes

The external balance derived indirectly from analysis of the slices. The slices removed from the chilled medium contained 78·4 % of water (S.D. ± 1·5 in 18 experiments), and had the ionic composition shown in Table 2. Comparison with the composition of the initial tissue (Table 1) suggests that, on chilling, the sodium in the tissue increased by 13 m-equiv/kg and the potassium decreased by 19 m-equiv/kg; chloride increased by 10 m-equiv/kg. These changes are not easy to interpret, because the amount of water had also increased. In terms of concentrations in the tissue water, sodium increased by 11 and chloride by 8 m-equiv/kg while potassium decreased

by 27 m-equiv/kg. The fall in *concentration* of potassium was thus greater than the increase in the *concentration* of sodium. The results expressed in terms of dry matter presumably indicated changes in the *amounts* of the ions in the tissue rather than in their concentrations. They indicated that the amount of sodium gained (124 m-equiv/kg dry matter) was more than twice the amount of potassium lost (53 m-equiv/kg dry matter). The gain of chloride (94 m-equiv/kg dry matter) was comparable with that of sodium.

TABLE 2. Composition of slices removed from chilled medium

(Averages and standard deviations from 18 experiments)

	m-equiv/kg tissue	m-equiv/kg dry matter	m-equiv/kg water
Sodium	105 ± 4	490 ± 41	134 ± 6
Potassium	36 ± 3	165 ± 12	46 ± 4
Chloride	82 ± 8	385 ± 51	105 ± 9
	$(Na^+)+(K^+)$		180 ± 7
	$(Na^+)+(K^+)+Cl^-)$		285 ± 11

The external balance calculated from changes in the amount and composition of the medium. It has already been mentioned that the volume of the medium was reduced by uptake of fluid into the slices. The most striking change in the composition of the medium was an increase in the concentration of potassium from an initial 5 m-equiv/l. to a final concentration which ranged from 9·3 to 16·5 m-equiv/kg in the individual experiments and averaged 12·1 m-equiv/kg (S.D. ± 2·4 in 18 experiments). Final concentrations of sodium ranged from 134 to 150 with a mean of 144 m-equiv/kg (S.D. ± 6), which did not differ significantly from the initial concentration of 145 m-equiv/l. There was no significant change in the concentration of chloride; final values ranged from 135 to 155 m-equiv/kg, with a mean of 143 (S.D. ± 6).

In order to calculate the external balances of the tissue from the changes in the medium, the amount of medium remaining in the tube at the end of each experiment was taken to be the weight actually removed for analysis plus the increase in weight of the small piece of filter paper used to blot the slice. This whole amount of medium was assumed to have the same composition as the major part of it which was actually analysed. The amount of dry matter in the slices at the beginning of the experiment was required in order that balances might be expressed in terms of dry matter, but it could not be determined directly. It was therefore taken to be the amount found by drying the slice removed at the end of the experiment, plus one-tenth of the weight of debris which remained in the tube after slice and medium had been removed. Table 3 shows the amounts of sodium and chloride which left the medium, and the amounts of potassium which appeared in the medium, expressed both per unit of initial moist weight

and per unit of dry matter as just defined. It is of interest to compare these changes in the amounts of electrolytes in the tissue with the differences between the compositions of the initial tissue and the chilled slices (Tables 1 and 2). These differences are shown in Table 4 to facilitate comparison.

Tables 3 and 4 show no agreement between results expressed in terms of moist tissue, but fair agreement when the exchanges were expressed in terms of dry matter. The results derived from changes in the medium, no matter how they were expressed, showed that the amount of sodium gained by the tissue was rather more than twice the amount of potassium lost.

TABLE 3. Exchanges between previously equilibrated kidney slices and chilled medium. Uptake of ions by tissue during immersion in the cold, derived from alterations in the medium and referred to moist and to dry weights of slices. (Averages and standard deviations from 18 experiments)

	m-equiv/kg initial tissue	m-equiv/kg dry matter
Sodium	$+33 \pm 10$	$+139 \pm 42$
Potassium	-15 ± 3	-60 ± 8
Chloride	$+26 \pm 17$	$+104 \pm 63$

The − signs indicate loss of potassium from slices to medium.

TABLE 4. Differences in composition between initial (warm-equilibrated) and chilled kidney slices (Tables 1 and 2), showing increases in concentrations of ions during immersion in the cold

	m-equiv/kg initial tissue	m-equiv/kg dry matter
Sodium	+13	+124
Potassium	−19	−53
Chloride	+10	+94

The − signs indicate that the concentration of potassium decreased.

This important result is obscured by comparing the analytical figures for moist tissue, which show alterations in concentrations rather than in amounts of ions. The changes in the medium resulted from alterations in the amounts of the various ions in the tissue, and these alterations were also reflected in the results of direct analysis of the tissue, provided that these were expressed in terms of dry matter, as in column 2 of Table 4. Precise agreement between the figures in the second columns of Tables 3 and 4 was not to be expected. The figures are not quite comparable because some of the sodium which left the medium appeared in debris and some of the potassium which appeared in the medium came from debris, whereas the net exchanges shown in Table 4 were derived from analysis of the slices only.

The tonicity of the fluid exchanged with the slices during swelling

The sum of the concentrations of sodium and of potassium in the water of the slices as used (Table 1) was 196 m-equiv/kg, considerably greater than the corresponding sum (150 m-equiv/l.) in the medium. In slices which had been chilled the total concentration of sodium + potassium had fallen from 196 to 180 m-equiv/kg water (Table 2). This difference in concentration was not large, but it was highly significant ($t = 4{\cdot}92$; $P = 0{\cdot}000001$), and might suggest that the fluid which had entered the slices when they swelled was more dilute than the medium. The total concentration of sodium, potassium and chloride, however, showed a smaller change, from 293 m-equiv/kg water initially (Table 1) to 285 m-equiv/kg water in the chilled tissue (Table 2). This smaller difference was not significant ($t = 1{\cdot}49$; $P = 0{\cdot}15$ approx.), partly on account of the greater variance of the determinations of chloride.

It has already been mentioned that the medium showed an increased concentration of potassium which was not offset by a reduction in the concentration of sodium or of chloride. After immersion of the slices in the cold, the sum of the concentrations of sodium and potassium in the medium recovered from the tubes averaged 157 m-equiv/kg medium (S.D. $\pm$ 4 in 18 experiments), compared with 150 m-equiv/l. for the medium initially. The average total concentration of sodium, potassium and chloride in the medium recovered after immersion was 299 m-equiv/kg medium (S.D. $\pm$ 4 in 18 experiments) compared with 290 m-equiv/l. initially. These differences are highly significant and support the suggestion that the fluid which entered the slices was more dilute than the medium.

The concentration of electrolyte in the fluid taken up by the slices was estimated by dividing the net gain of ions in each of the 18 experiments by the amount of fluid which left the medium. The results varied rather widely from one experiment to another. For the net exchange of sodium and potassium, the extremes were 4 and 195 m-equiv/kg of entering fluid. The fluid taken up appeared more concentrated than the medium in 3 and more dilute than the medium in 15 experiments. Its average concentration computed for sodium and potassium was 82 m-equiv/kg (S.D. $\pm$ 54), little more than half the concentration of the medium. The net exchange of sodium, potassium and chloride averaged 191 m-equiv/kg of fluid taken up (S.D. $\pm$ 67) about two-thirds of the total concentration of the same three ions in the medium. The variation between experiments was again large, ranging from 36 to 337 m-equiv/kg. The entering fluid appeared more concentrated than the medium in one experiment and more dilute in 17. Although nothing is known about organic constituents, it can be concluded that the fluid which entered the slices when they were chilled was sub-

stantially more dilute than the medium in its principal inorganic constituents. This conflicts with the findings of Leaf (1956). Some earlier experiments, carried out without preliminary equilibration of the slices with the warm oxygenated medium, yielded results in agreement with those of Leaf, and will therefore be reported briefly here.

Exchanges between fresh tissue and a chilled medium

Freshly-cut kidney slices were lightly blotted and then placed immediately in the small incubating tubes without previous contact with the medium. Apart from the omission of the preliminary equilibration with the warm, oxygenated medium, the slices were treated as in the main experiments, but chloride was not determined.

TABLE 5. Composition of fresh kidney slices as cut and after immersion in chilled medium

(*a*) Initial composition. (Averages and standard deviations from analysis of 12 samples)

	m-equiv/kg tissue	m-equiv/kg dry matter	m-equiv-kg water
Sodium	61 ± 4	252 ± 17	81 + 6
Potassium	82 ± 8	337 ± 23	108 ± 12

(*b*) Final composition. (Averages and standard deviations from 20 experiments)

	m-equiv/kg tissue	m-equiv/kg dry matter	m-equiv/kg water
Sodium	100 ± 9	531 ± 49	123 ± 11
Potassium	41 ± 4	218 ± 16	51 ± 5

Changes in composition of the tissue. The slices as cut contained 75·8 % of water (S.D. ± 1·1 in 12 analyses). After 1 hr in the chilled medium, swollen slices contained 81·0 % of water (S.D. ± 1·1 in 20 experiments). Table 5 shows the ionic composition of the fresh and of the swollen tissue. The final composition after chilling was not vastly different from that of the slices which had been submitted to preliminary washing in the warm oxygenated medium (Table 2). Comparison of Tables 1 and 5(*a*), however, shows that the tissue had gained sodium and lost potassium during the preliminary treatment, so that fresh slices lost more potassium and gained more sodium when they were chilled. Tables 6 and 7 allow the effects of chilling upon the composition of the slices (expressed both in terms of moist and of dry weight) to be compared with the net exchanges of ions deduced from alterations in the amount and composition of the medium. As in the experiments upon slices which had been previously equilibrated with the warm, oxygenated medium, the net exchanges deduced from alterations in the medium agreed rather closely with differences in composition in terms of dry matter. Differences in composition

based on moist weight obscured the fact that the amount of sodium gained was far greater than the amount of potassium lost.

Changes in the medium. The greater loss of potassium from the fresh slices, and their greater uptake of sodium, caused larger alterations in the composition of the medium than had been seen in the other group of experiments. The final concentration of potassium in the medium averaged 19·3 m-equiv/kg (S.D. ± 3·5 from 20 experiments). The increase of 14·3 m-equiv/kg in the concentration of potassium was offset by a fall in the concentration of sodium from 145 m-equiv/l. in the initial medium to an

TABLE 6. Exchanges between fresh kidney slices and chilled medium. Uptake of ions by tissue during immersion in the cold, derived from alterations in the medium and referred to moist and to dry weights of slices. (Averages and standard deviations from 20 experiments)

	m-equiv/kg initial tissue	m-equiv/kg dry matter
Sodium	+63 ± 21	+300 ± 68
Potassium	−27 ± 7	−119 ± 24

The − signs indicate loss of potassium from slices to medium.

TABLE 7. Differences in composition between fresh and chilled kidney slices (Table 5) showing increases in concentrations of ions during immersion in the cold

	m-equiv/kg initial tissue	m-equiv/kg dry matter
Sodium	+39	+279
Potassium	−41	−119

The − signs indicate that the concentration of potassium decreased.

average final concentration of 132 m-equiv/kg (S.D. ± 5). The sum of the concentrations of sodium and potassium was therefore 151 m-equiv/kg (S.D. ± 4), which did not differ appreciably from the sum in the original medium. The fluid taken up by the fresh slices thus appeared to have the same concentration as the medium. This was confirmed by the average value of the fraction,

$$\frac{\text{Na leaving medium} - \text{K appearing in medium}}{\text{amount of medium disappearing}},$$

which was 130 m-equiv/kg (S.D. ± 24), and so hardly differed from the concentration of monovalent cations in the medium.

A note on the composition of the debris

Fifteen pooled samples of debris from slices which had first been equilibrated with warm, oxygenated medium and then immersed in small volumes of medium at 0° C were analysed for sodium and potassium, eight of these for chloride also. The moist debris had the composition

indicated in the first column of Table 8. It is difficult to assign a precise water content to the debris because, even after it had been centrifuged to form a fairly hard pellet, a variable amount of the supernatant medium must have remained with it. Calculations based upon the approximate figure of 90 % of water by weight, which was found by drying a small number of specimens, gave the figure in the second and third columns of Table 8. These results suggest that, even in the cold, the debris accumulated sodium, potassium and chloride in concentrations higher than those in which the same ions were present in the supernatant medium. Moreover, the ratios of Na^+:K^+ in the debris and in the medium differed, which might suggest that the debris contained a substance possessing a selective affinity for alkali-metal ions. Such a substance might be expected to occur in the cells of an organ concerned with the transport of these ions.

TABLE 8. Composition of debris left in tubes after removal of slices and medium. (Averages and standard deviations are from determinations of Na and K on 15 samples, and of Cl on 8 samples)

	m-equiv/kg debris	m-equiv/kg dry matter*	m-equiv/kg water*
Sodium	168 ± 15	1680	187
Potassium	28 ± 5	280	31
Chloride	147 ± 21	1470	163

* Assuming that the moist debris contained 90 % of water.

DISCUSSION

The present method permits exchanges of ions and of water between a single tissue slice and the surrounding medium to be determined directly. The most valuable result of its application has been the confirmation that differences in tissue composition, if expressed in terms of dry matter, truly reflected the shifts of ions between tissue slices and the medium under the conditions of the present experiments. Two important conclusions, that the amount of sodium entering the swelling tissue when its metabolism was depressed greatly exceeded the amount of potassium lost, and that chloride accompanied sodium into the tissue, had hitherto been deduced indirectly from differences in composition between separate pieces of tissue. They have now been confirmed by the changes in the medium which accompanied the swelling of a single piece of tissue. The extent of the entry of water into the tissue was also confirmed by its disappearance from the medium. The present results are in general agreement with the conclusions of Mudge (1951) and of Leaf (1956, 1959) that the uptake of sodium and chloride by the tissue is probably the most important cause of the imbibition of water during swelling.

The question, whether the whole of the swelling can be interpreted as an isosmotic transfer of water accompanying sodium and chloride taken up

by the tissue, is left open. Leaf (1956) claimed that the fluid taken up was isotonic with the medium. This was only found in the present work in experiments which, like those of Leaf, involved comparing freshly-cut tissue, which had not been incubated at all and still contained blood and tubular urine, with slices incubated in the cold. The more satisfactory experiments, in which slices freed from blood and tubular urine were transferred from warm to chilled medium, revealed a comparable degree of swelling, but the fluid taken up was distinctly less concentrated than the medium in respect of its major inorganic constituents.

Autolysis might help to explain the apparent hypotonicity of the fluid taken up during swelling. If, but only if, the products of autolysis had remained inside the cells, water should have been taken up out of proportion to inorganic solutes, and the medium should have been left more concentrated in these, as was in fact found. The experiments of Leaf (1956) were made with slices from the kidneys of guinea-pigs; and Conway, Geoghegan & McCormack (1955) reported that renal tissue from guinea-pigs, unlike that from rats, did not undergo autolysis, at least when kept at 0° C after grinding in the frozen state. The possibility that autolysis contributed to the swelling observed in the present experiments cannot be ruled out, because organic constituents were not determined, so that it was not possible to draw up a complete osmotic balance sheet. But autolysis does not provide so plausible an explanation of swelling as might appear at first sight (cf. Robinson, 1960). Swan & Miller (1960) have recently adduced further evidence that products of autolysis did not remain in the cells, and played no part in the swelling of tissue slices when metabolism was depressed. In their experiments, however, in which shifts of ions were deduced from the composition of the tissue, the fluid which entered the slices never seemed to be less concentrated than the medium.

It may be concluded that movements of water in the present experiments were partly independent of shifts of the principal inorganic constituents of the tissue and medium. It still remains to determine what other factors were involved.

SUMMARY

1. Slices of cortical tissue from the kidneys of adult rats were immersed in small volumes of a saline medium at 0° C.

2. Net exchanges of water, sodium, potassium and chloride were determined from the changes in amount and composition of the medium, and compared with exchanges deduced from the differences in composition between the slices as used and those which had been immersed in the chilled medium.

3. Sodium and chloride disappeared from the medium in comparable

amounts, which were more than twice as great as the amount of potassium which the medium gained from the slices.

4. These net exchanges were well reflected in the differences in composition between chilled and starting tissue, provided that composition was expressed per unit of dry matter.

5. The water which disappeared from the medium was recovered in the swollen slices and in a small amount of debris left in the tubes.

6. The fluid which entered the tissue during swelling was less concentrated than the medium in respect of its major inorganic constituents.

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NET DEPOLARIZATION AND DISCHARGE RATE OF MOTONEURONES, AS MEASURED BY RECURRENT INHIBITION

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Sherrington, struck by the way reflex excitation and inhibition could be graded against each other, often used the simile that this took place as if by algebraical summation. To-day it is possible to translate this general notion into the simplest kind of algebra and find out how well it works in suitable experiments. Such an attempt forms the subject of the present paper. Recurrent inhibition (Renshaw, 1946) of tonically discharging extensor motoneurones in mammals has been used because this acts by the antidromic route and hence can be made maximal and constant with less risk of stirring up accessory circuits than is present with orthodromic inhibitions.

We have previously reported (Granit & Rutledge, 1960; Granit, Haase & Rutledge, 1960) that the normal tonic firing frequency F_n of an extensor motoneurone in the decerebrate preparation is depressed to a slower rate F_i under maintained recurrent inhibition. F_i was found to be linearly related to F_n. In those experiments the antidromic stimulus was locked to the discharging spike and thus was forced to act at the low average frequency of the latter. Therefore it varied with the discharge rate of the motoneurone to whose action it was tied, the experiments being designed to study 'frequency limitation' of a discharge. The present problem requires that antidromic stimulation should be constant in rate and strength and of high frequency while the rate of firing of the motoneurone (F_n) is varied by tetanizing extensor muscle nerves of de-efferented animals at different rates and strength. It will often become necessary to draw upon the results of the two papers mentioned above, which will then for convenience be referred to respectively as (I) and (II).

Since the experiment is run in terms of discharge frequency it is necessary to know how this is related to depolarizing current across the cell

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membrane. Direct measurements by K. Frank and M. G. F. Fuortes (personal communication) prove the relationship to be linear over a range greatly exceeding the normal firing frequency of the motoneurones. Thus the concept 'generator potential' not only applies to the motoneurones but, just as in several sense organs (Katz, 1950; MacNichol, 1956; Fuortes, 1958, 1959; Loewenstein, 1960; Wolbarsht, 1960), impulse frequency is likely to be proportional to magnitude of generator potential, even though at the moment the evidence for this view is based merely on artificial stimulation of motoneurones by the electrical current.

Motoneurones differ from sense organs in being simultaneously bombarded by both excitatory and inhibitory impulses. For the purpose of the test we shall accept the now well-supported conclusion from the work of Eccles and his colleagues (see e.g. Eccles, 1957, 1961) that excitation and inhibition have their equivalents in depolarizing and polarizing potentials across the cell membrane corresponding to depolarizing and polarizing currents. This by no means excludes other forms of inhibition, but these at the moment do not concern us since the recurrent variety is of the polarizing type (Eccles, Fatt & Koketsu, 1954). From this would follow (cf. I, II) that the discharge frequency

$$F = k(P_{dep} + P_{pol}), \tag{1}$$

where F is the discharge frequency of the motoneurone, k a constant, P_{dep} the excitatory and P_{pol} the inhibitory current, the two being of opposite sign. Their difference $(P_{dep} + P_{pol})$ is defined as 'depolarizing pressure' (cf. I; and Phillips, 1959) or, more strictly, as net depolarizing current.

If now a motoneurone is excited by maintained orthodromic tetanization of the muscular afferents to discharge at various rates F_n, an antidromic tetanus of constant rate and strength, causing a constant inhibition (P'_{pol}), should, since

$$F_n - F_i = -kP'_{pol}, \tag{2}$$

reduce the discharge frequency by a constant number of impulses.

The derivation of eqn. 2 is based on the effect of current applied to the motoneurone and on what has been found by studying motoneurones with the intracellular technique during recurrent inhibition (cf. above). It is not evident that it is valid for a reflex in which, for instance, P'_{pol} may be influenced by the orthodromic stimulus (cf. paper II, and Haase & Van Der Meulen, 1961). The aim of the present work is to find out by experimentation whether the generalization made in eqn. 1 (and its corollary eqn. 2) is legitimate.

Observations have also been made on the relation between $F_n - F_i$ and antidromic tetanus rate. Finally, the results have been considered

from the point of view of the tasks assignable to the recurrent collaterals which are common in all nervous centres.

METHODS

The decerebrate cat was used and single ventral root fibres were functionally isolated in $L7$ or $S1$. Both legs were denervated and on the experimental side the ventral roots were severed intradurally from $L5$ to end of cord. Sometimes the tail end of the cord was cut across (when the tail tended to move). Stimulating electrodes (Fig. 1) were placed on the central ends of the severed gastrocnemius nerves to elicit a maintained tonic reflex by tetani varied in rate and strength (cf. I, II). Another pair of electrodes was placed on the

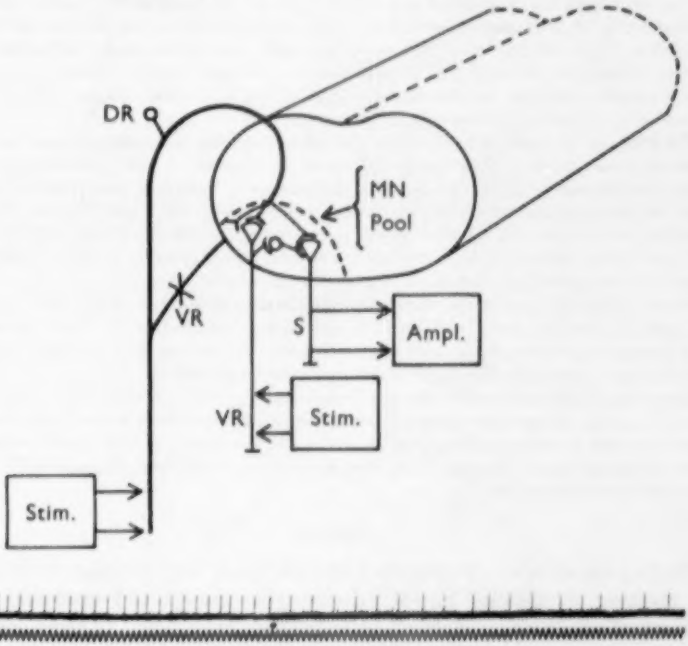

Fig. 1. Experimental arrangement. Stimulating electrodes on severed gastrocnemius nerves and on ventral root (VR) from which single fibre (S) has been isolated. DR, dorsal root. Within motoneurone pool (MN) recurrent circuit diagrammatically shown. Below, record of spike discharging in response to repetitive stimulation of gastrocnemius nerves; antidromic stimulation at 48/sec inserted at moment marked by dot; time marker, 100 c/s.

rest of the ventral root from which the fibre was isolated (see Fig. 1) in order to stimulate its fellow motoneurones antidromically so as to induce recurrent inhibition. In any one experiment the rate of antidromic tetanization was of constant and maximal strength, its rate being not less than 42/sec and generally between 55 and 65/sec. This reduced the firing frequency of the motoneurone from F_s to the inhibited discharge rate F_i.

The decerebrate animal, being 'tonic' in itself, offers the advantage of making it possible to stay within low stimulus strengths in trying to increase F_n. This is of some importance because repeated use of strong tetani tends to decrease stability of the recurrent effect (I, II). Such stimuli might influence the Renshaw cells as proved for dorsal root stimulation by Haase & Van Der Meulen (1961). Eccles *et al.* (1954) have found no effects on Renshaw cells from large muscular afferents. Our problem required finding motoneurones which could be made to discharge over a large range. The great majority of tonic cells is heavily stabilized in rate of discharge with respect to muscular afferents (Denny-Brown, 1929; Granit, 1958; I; II). To obtain a number of cells varying in F_n over a large range it became necessary to study some less well stabilized cells during the adaptive phase characterized by gradually diminishing discharge rate.

In this paper we have called 'Procedure I' the earlier type of experiment (II) in which the recurrent inhibition is inserted at a definite moment, in this case 20 sec after initiation of stimulation. F_n is measured for 4–5 sec before and after the period of 5 sec antidromic stimulation to give F_1. The two F_n values are averaged. Such measurements are single tests repeated at intervals of 1 min. The disadvantage of Procedure I is that it takes a long time to accumulate a sufficient number of values, thus giving systematic changes in the state of the animal a chance to influence the measurements.

'Procedure II' consists in maintaining the afferent tetanus for some time and by automatic regulation inserting 2 sec antidromic tetani at intervals of 7 sec. Between each run 2 min were allowed to elapse. Procedure II gave access to 'adapting' motoneurones whose initial frequency accordingly was high relative to the final stable value. The risk with this procedure is that available surplus excitation in many such cells is reduced while stimulation goes on, as analysed in (I), and that high rates of F_n are more favourably located with respect to this time factor than are low rates. To some extent this systematic error can be overcome by taking runs at low stimulus strengths also, giving low values of F_n a chance to appear in the beginning, and also by multiplying the number of observations. For F_n, 2–4 sec measurements before and after antidromic stimulation were averaged and some three to five values were obtained for each run. Independently of discharge rate, time of maintenance of a discharge will in many cells influence $F_n - F_1$. A description of such effects was given in (I). Some cells do not possess this complication, which makes it necessary to restrict number of tests in each run by Procedure II. We have also made combined experiments applying both Procedures I and II to the same motoneurone. This should be a good check on systematic errors.

RESULTS

Testing equation 2. A graphic presentation of our findings is obtained by plotting F_1 against F_n in impulses per second and studying some individual experiments before summarizing the results. If there were no recurrent inhibition acting upon the selected motoneurone, F_n would be identical with F_1 and the plot of one against the other a straight line with an angular coefficient of 45°. This line is drawn in all the records of Fig. 2. Equation 2 then states that, in every case where recurrent inhibition is present, the curve obtained must be parallel to this line and shifted downwards by an amount dependent upon the degree of recurrent inhibition in that particular motoneurone. It follows from eqn. 2 that the regression line, as calculated for each motoneurone by the method of least squares, in an ideal case should be at 45° from the x-axis, i.e. possess a regression coefficient $b = 1{\cdot}00$. The coefficients for the samples (= all observations

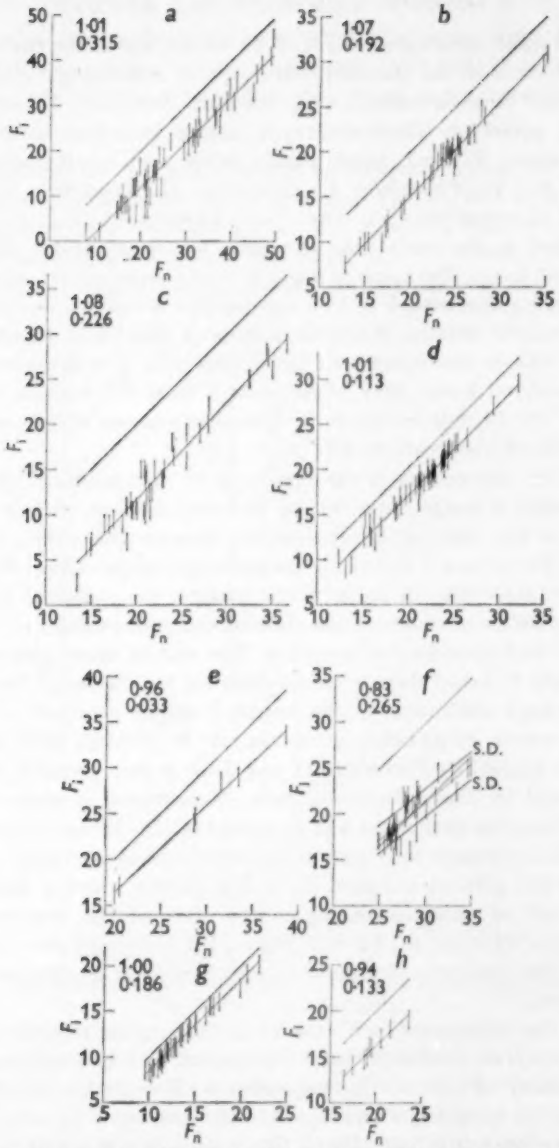

Fig. 2. Eight different experiments aiming at measuring $F_n - F_i$. A 45° line drawn in each diagram to show $F_i = F_n$. Regression line, regression coefficient and standard error shown in each graph, the two latter on the left; standard deviation shown in the graph (*f*) in which range is small and variability large. In this case second line crossing regression line is drawn to regression coefficient 1·00. *a*, Procedure I+II; *b*, same; *c*, Procedure II; *d*, Procedure I+II; *e*, Procedure I; *f*, Procedure II; *g*, same; *h*, Procedure I.

on any one spike) illustrated in Fig. 2 are shown above the curves in each graph together with the standard error $s/\sqrt{N}$ in measuring $F_n - F_1$; N here is the number of observations, s the standard deviation. These examples have been chosen to illustrate experimental variations such as large range (F_n max $- F_n$ min), small range, large and small values of the difference $F_n - F_1$, Procedure I, Procedure II, Procedure I+II, and number of observations (N). One 'bad' experiment (Fig. 2(f)) is also included, bad in the sense that the range was small and s (marked S.D in the graph) large. The value of b was 0·83 in this case, but, considering s the alternative line with $b = 1{\cdot}00$ can hardly be said to be excluded by the experimental results. While deviations of individual regression lines from $b = 1{\cdot}00$ do not necessarily invalidate eqn. 2, it is nevertheless of some interest to know that while only 2 cells fell outside the limits $0{\cdot}9 < b < 1{\cdot}1$, 16 cells fell inside it. The total number of cells was 18, the total number of observations 470.

Particularly interesting is the rare type of motoneurone (cf. Fig. 2a) which covered a range from 9·0 to 49·5 impulses/sec with a recurrent inhibition of the order of 8 impulses/sec, because this neurone could be studied by Procedures I and II for low and high-range values; 58 measurements were obtained. In spite of the scatter, the standard error being 0·315, the number of observations allowed the coefficient b (= 1·01) to be determined with considerable accuracy. The scatter arose from the values by Procedure II taken during 'adaptation' of the discharge frequency to the small range obtainable by Procedure I (which generally is more reliable). However, range being important for the problem, half the number of cells was studied by Procedure II and three in that group by both Procedures I and II. The influence of time of maintenance of the discharge and loss of surplus excitation was discussed in (I). When in spite of constant F_n the resistance to recurrent inhibition changes rapidly, the cell is useless for the present purpose. It is not known whether this depends upon the cell or upon something in the state of the preparation. Of 20 cells only 2 were left out for this reason. The beginning and end of every discharge offer problems of their own, which will not be considered in the present work.

Now all the experiments with the 18 motoneurones, regardless of range and frequency of discharge, may be treated as one single experiment testing validity of eqn. 2. The regression coefficients for individual cells should then be weighted with respect to the number N in order to obtain the true average value for b. When this is done it is found that

$$\Sigma(bN)/\Sigma(N) = 0{\cdot}996$$

which is an unexpectedly good approximation to the theoretical, $b = 1{\cdot}00$.

For each cell or sample the standard error ($s/\sqrt{N}$) for measuring $F_n - F_1$ was calculated (see below and Fig. 2). The average is 0·240. The mean $F_n - F_1$ is 4·86, the weighted mean 5·45. This means that in the averages

$$F_n - F_1 = 5{\cdot}5.$$

Still regarding the 470 observations as members of one single experiment devoted to examining the constancy of $F_n - F_1$, it is of considerable interest to study the deviations from the mean $F_n - F_1$ for each motoneurone. The average standard deviation was 1·14 and experimental experience shows that the values always can be measured to 1·0 spike/sec.

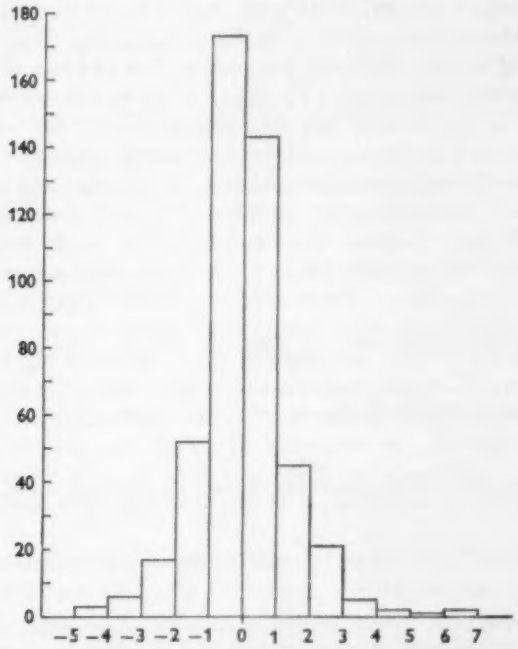

Fig. 3. Histogram of 470 deviations from $F_n - F_1$ = constant. Unit variate for the classes is 1·0 impulse/sec.

Therefore, in plotting a histogram in order to demonstrate the distribution of the deviations from the sample means, the unit variate is chosen to be 1·0. Figure 3 illustrates the result. There is no reason to believe that the distribution of variates is anything but normal. Apparently systematic errors are of a small order and have cancelled out. Thus the results of the analysis of Fig. 3 agree with the findings based on the idea of an average regression coefficient of the order of 1·0.

The normal distribution in Fig. 3 means that it is legitimate to calculate the limits of error of the sample means for their various degrees of freedom. This calculation has been carried out for the probability level 0·95 and consists in multiplying the standard error by the appropriate factor t. It is believed that after giving for each experiment the value so obtained together with N (in brackets), further elaboration of the results by statistical methods yields nothing new, even though for some of the best experiments the variance around the regression line was also calculated. For the 18 cells $ts/\sqrt{N}$ is: 0·15 (12), 0·19 (7), 0·23 (22), 0·59 (18), 0·52 (9), 0·63 (10), 1·34 (8), 1·26 (14), 0·31 (9), 0·63 (58), 1·02 (24), 0·45 (44), 0·23 (48), 0·39 (39), 0·31 (42), 0·54 (41), 0·18 (30), 0·38 (35), mean = 0·519.

Variation of rate of antidromic stimulation. The problem of $F_n - F_1$ as a function of antidromic stimulus frequency is not as well defined as that of the previous section because the discharge pattern of the Renshaw cells changes from the well-known bursts at slow rates to single spikes per shock at high rates of tetanization and the transitional region is not precisely known *in casu*. Parallel work in this laboratory by J. Haase & J. Van Der Meulen (1961 and personal communication) on single Renshaw cells established that the probable range within which this change takes place is from 5 to 15 shocks/sec. From our point of view rates below 5/sec are of little physiological interest, because motoneurones do not discharge rhythmically below 5/sec and many of them have still higher thresholds for regular repetitive firing (see statistical distribution curves in paper II as well as the actual measurements shown above in Fig. 2). An effect of stimulus frequency on the value of $F_n - F_1$ can generally be traced up to 30/sec, sometimes to 40/sec (see Fig. 5); this range is also the characteristic range of variation of the discharge frequencies of motoneurones.

The physically well-defined stimulus frequency is thus changed by the intercalated Renshaw cell into a function which for low rates cannot increase in proportion to the number of antidromic shocks per second. A simple calculation also exemplifies this: let slowly repeated antidromic shocks be followed by silent periods of the order of 40 msec—a value typical of many motoneurones. If these silent periods could be maintained up to stimulus rates of the order of 25/sec and if F_n were of the same order, then recurrent inhibition ought to be practically permanent, i.e. $F_1 = 0$. This is never the case.

Figure 4 should be studied from these points of view. As antidromic stimulation rates increase, irregular silent periods are gradually replaced by an 'inhibitory state' of reasonably regular firing at some rate lower than that of the control. The two experiments have been chosen to demon-

strate one case with, and one without rebound. Clearly, at low rates of antidromic stimulation, the momentary increase of discharge frequency during rebound compensates for the preceding loss of spikes in the silent

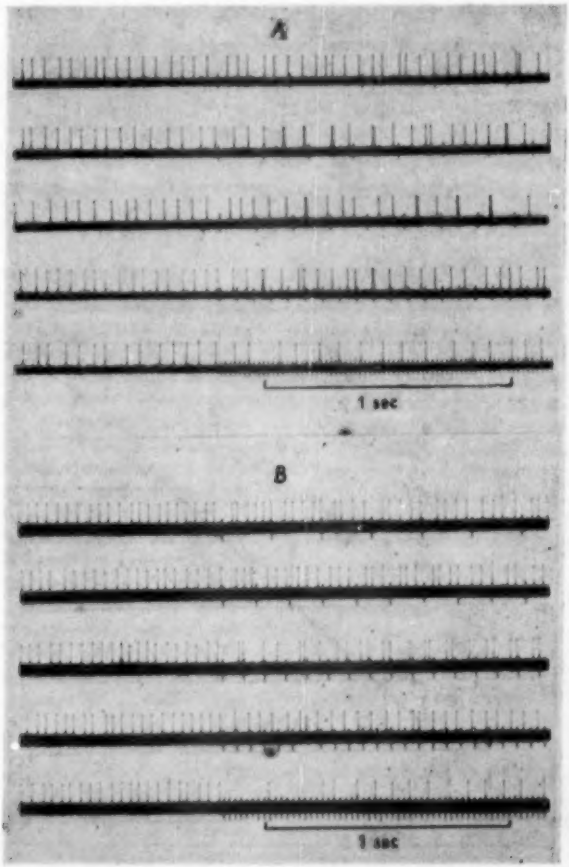

Fig. 4. Two different experiments in which rate of antidromic stimulation is varied from record to record. Stimulus frequencies from above downwards are: (shocks/sec) *A*, 3·4, 5·5, 6·7, 14·5, 48·0; *B*, 4·8, 7·4, 11·6, 17·4, 42·0.

periods. We believe this rebound to be a useful index (when present) of the transformation of the pattern of discharge of the Renshaw cells, as discussed above. Rapidly occurring antidromic shocks cut short incipient rebound and replace it by a fresh wave of recurrent inhibition.

After these remarks, which show that a variation of stimulus rate introduces one more variable into an already difficult experiment, it may seem surprising that in the few experiments in which complete curves were obtained the results suggested that (beginning from such low rates as

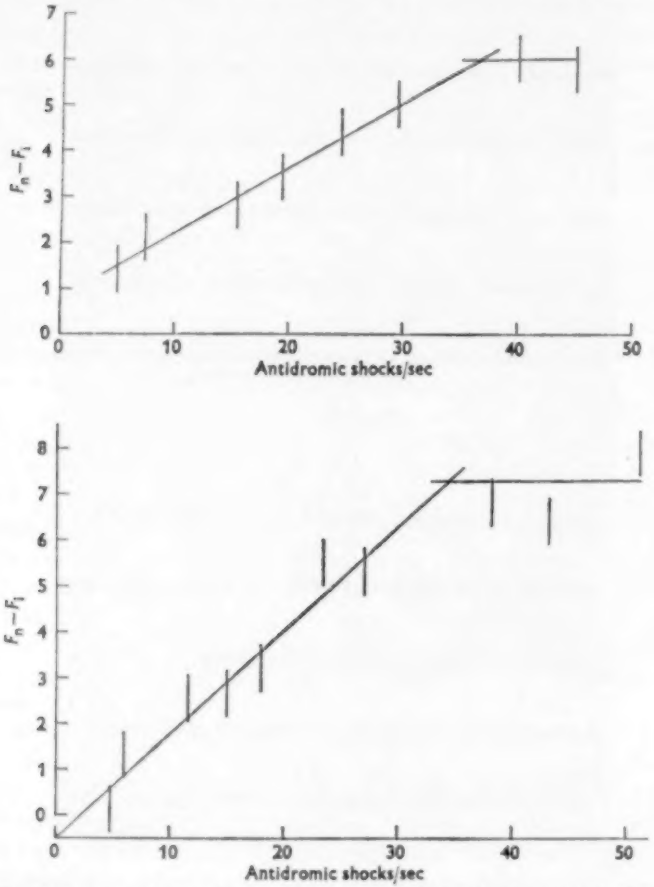

Fig. 5. Two experiments showing effect of antidromic stimulus rate (abscissa) on $F_n - F_1$. The horizontal line shows final value of the latter.

5–10 shocks/sec) up to the maximum $F_n - F_1$, efficacy of recurrent inhibition actually increased in direct proportion to frequency of antidromic stimulation. This is illustrated in Fig. 5. The curves had a flat portion at very slow stimulation rates (not illustrated) which represents the threshold for the effect of stimulus frequency, when only irregular silent periods

and rebounds are produced by the antidromic shocks. These experiments also show why it was necessary to use an antidromic tetanus of high frequency.

It should be pointed out that the bursts by which Renshaw cells discharge at slow rates of antidromic stimulation are very likely artifacts from the physiological point of view, except with well synchronized single contractions, convulsions etc. Some motoneurones (see Fig. 4*B*) show signs of this initial burst by having a long initial pause. Others do not. The Renshaw cells are naturally stimulated by the asynchronous firing of the many motoneurones which converge upon them and not by slow synchronous shocks. For this reason the linearly rising portion of the curves of Fig. 5, falling as it does within the range within which motoneurones discharge, is probably a better approximation to something normal than the effects seen at low rates in Fig. 4.

In our previous experiments (II) when the antidromic shock was tied to the firing spike its frequency varied with the low discharge frequency of the motoneurone investigated. As a consequence one would have expected $F_n - F_i$ always to increase with F_n, as it actually did in many experiments and on an average (33 motoneurones), confirming the results of Fig. 5. But in those experiments there were several individual cases in which $F_n - F_i$ was maximal at low rates of discharge (= low rates of antidromic stimulation) and decreased for higher rates. At the time it was suggested that this was due to competition with 'natural' recurrent inhibition. The present results suggest, however, that owing to the low discharge rates and small frequency range of the previous experiments (12 impulses/sec on an average; F_n min. forming a peak between 6 and 10 impulses/sec), several tests must have fallen within the transitional range where the discharge pattern of the Renshaw cells undergoes large alteration, and that this is a more likely explanation of the exceptions mentioned, inasmuch as at slow rates bursts from the Renshaw cells occur (cf. above) and extend duration and thereby efficacy of inhibition. In the first set of experiments of this paper the rate of antidromic stimulation was constant, generally around 55/sec, and so the experiments cannot very well be compared with the earlier ones in which rate of stimulation was at the low and variable firing frequency of the motoneurones.

DISCUSSION

The deduction tested in this paper (eqn. 1) extended to reflex firing what seemed a likely consequence from work on direct electrical stimulation of motoneurones (K. Frank & M. G. F. Fuortes, 1960, and personal communication) and on intracellular recording of changes of membrane potential during recurrent inhibition (Eccles *et al.* 1954). The results have been in good agreement with our deduction. The test carried out would seem to be a necessary preliminary to any quantitative work on motoneurones enagaged in balancing out the net effect of natural excitatory and inhibitory influences converging upon them. In a general way our conclusions are also supported by the qualitative findings of Phillips (1956) and Terzuolo (1959). They may further be said to extend the realm of applicability of the concept of 'generator potential' (Granit, 1947).

The biological law established here—like all such laws—can hardly be valid in the extremes. At the low rates or strengths of stimulation needed in many motoneurones for small values of F_n, there may not be enough surplus excitation to support the discharge. This problem was studied in detail by Granit & Rutledge (1960). At the highest rates of F_n the output from the fellow motoneurones, clashing as it does with the antidromic stimuli, may succeed in effectively reducing the rate of antidromic stimulation. Animals in feeble condition may never be able to support maintained, tonic discharges well enough for the test to be applied. Too strong afferent stimuli may alter the quantity P'_{pol} (cf. II; and Haase & Van Der Meulen, 1961) and thus invalidate the conditions under which the trial must be conducted. It is also possible that the motoneurone possesses degrees of freedom with respect to facilitation and depression which are not reflected by changes of membrane potential, such as effects of the electrical field (Fatt, 1957*a*, *b*) and unfathomed factors such as those described for invertebrates by Hagiwara & Bullock (1957), Bullock (1959) and Tauc (1960). However, the fact that the experimental test can be carried out at all, i.e. with cells remaining in reasonably steady states for the 1–2 hr required for making 30–50 observations—proves that our deduction contains an essential element of truth.

Of considerable interest is the detailed agreement with the results of Hartline (1949) and Hartline & Ratliff (1956) concerning the so-called lateral inhibition in the *Limulus* eye. This is linear with discharge frequency in the sense that one inhibiting ommatidium influences another adjacent one in proportion to its own rate of firing whilst the rate of firing of the inhibited ommatidium is unimportant as long as the inhibiting ommatidium is fired at constant rate. These are the very results we have had above with recurrent inhibition, which probably fulfils similar tasks, as suggested by Brooks (1959) and Wilson, Talbot & Diecke (1960). In our case the plan of work for the first experimental section was deducted from a hypothesis that possibly is valid for lateral inhibition also. In both cases frequency of discharge rather than spike interval gives simple linear relations. Hartline & Ratliff (1957) have designed interesting experiments calculated to throw light on visual mechanisms of contrast. In our case it is necessary to think of what the results signify for tonic reflexes.

To this end, let us recall that muscular contraction is by no means linear with frequency of efferent stimulation. On the contrary, low rates of stimulation (around 5/sec) have but little influence on the tonic soleus and then, as stimulus rate is increased, isometric tension rises rapidly towards a maximum reached asymptotically around rates of 30/sec. This is shown by the curve in Fig. 6 (black dots) taken from the work of Matthews (1959), which refers to soleus stimulated electrically through its nerve at

different rates. This is a muscle for which the natural frequency of efferent discharge in tonic reflexes is from 5 to 25 impulses/sec (Denny-Brown, 1929; Granit, 1958) and fairly independent of extension. Let us now assume that the scale on the abscissa represents a variation in firing rate from edge to centre (containing the most active neurones firing at 20–25/sec) of the soleus motoneurone pool. Let a constant recurrent inhibition, largely maintained by the centre of the pool, be distributed across this pool in accordance with our findings. This means that if from each abscissa 6 impulses/sec (average $F_n - F_1$ from p. 466) be subtracted, the upper curve (1) should illustrate how much the discharge across the pool would contribute to tension in the absence of recurrent inhibition. The difference

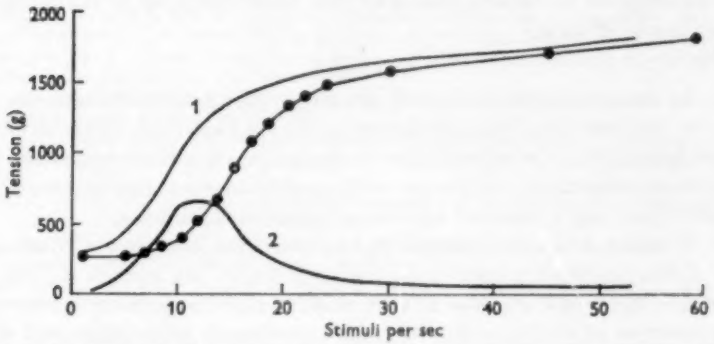

Fig. 6. Curve with observations in filled circles from P. B. C. Matthews (1959) illustrates isometric tension (ordinates) plotted against stimulus frequency to muscle nerve (abscissae) of soleus at initial length determined by tension value below 50 g.

It is assumed (see text) that abscissa also represents natural firing frequencies across the soleus motoneurone pool, the range being from 25 to 5 impulses/sec from centre to edge of pool. An approximate idea of what the experimental curve would have looked like in the absence of recurrent inhibition is obtained by subtracting for all points in the curve 6 impulses/sec. This is the upper curve (1). The lower one (2) is the difference between the two others.

between the two curves, drawn below them (2), shows the range of operation of recurrent inhibition on this assumption. Clearly its contribution to control of muscle tension has been most powerful in the very range in which the soleus motoneurones operate effectively. Furthermore, recurrent inhibition has served to diminish the contribution to muscular tension of the fringe neurones which fire at very slow rates. To this should be added that recurrent inhibition must be mutual, so that, on our results, the rapidly firing motoneurones will inhibit the slowly firing ones far more effectively than the latter can inhibit the fast ones. With an organization of the pool as implied in Fig.6, this means that the fringe neurones will

be further suppressed by their rapidly firing neighbours. Low discharge frequencies can on the whole never be maintained unless the corresponding motoneurones are very powerfully driven from the orthodromic side and thus capable of quickly replacing loss of depolarizing pressure (II). These factors also aid in concentrating the activity in the pool to its centre whose neurones are likely to be firing at rates capable of contributing decisively to muscle tension. Since recurrent inhibition is controlled from supraspinal stations (II; and Haase & Van Der Meulen, 1961) and since its effective range in soleus, according to Fig. 6, is also the critical range for changes of tension, recurrent inhibition should be a very potent instrument of control for tonic muscles. In the two previous papers (I, II) the significance of recurrent control has been discussed in detail from other points of view.

SUMMARY

1. In decerebrate de-efferented cats single-fibre reflexes in response to maintained tetanus of the gastrocnemius nerves have been recorded from appropriate ventral roots and their discharge rate has been suppressed by antidromic tetanization of the rest of the root at constant rate and strength thereby inducing a constant amount of recurrent inhibition.

2. If under such circumstances F_n be the normal frequency of discharge and F_i the inhibited value, both given in impulses per second, it is found that $F_n - F_i$ = constant. This relation was deduced from present knowledge of properties of motoneurones and the experiments were designated as a test of a hypothesis on these lines formulated in equations 1 and 2.

3. With respect to rate of antidromic tetanization at constant strength, it is found that $F_n - F_i$ is directly proportional to it from around 5–10/sec up to a maximum around 30–40 shocks/sec.

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ENCEPHALIC GLYCOGEN DIFFERENCES IN YOUNG AND ADULT RATS

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This paper is concerned primarily with the effects of pentobarbital anaesthesia and a metabolic stimulant, 2,4-dinitrophenol (DNP), upon two glycogen fractions of the cerebral hemispheres, diencephalon and cerebellum of young and adult rats. It has been accepted generally that the amount of glycogen in encephalic tissue is low, and recent evidence (Breckenridge & Crawford, 1960) indicates that synthesis in the brain from the uridinediphosphate glucose system is approximately six times less active than in other tissues. There appears to be some controversy as to the actual function of this polysaccharide in the brain, but Chesler & Himwich (1943) are firm in their belief that it is an intrinsic substrate utilized in both glycolysis and oxidation. A substantial amount of Russian work within the past decade has been directed toward brain glycogen changes during various functional and biochemical states of the animal. The opinion is offered by many of these investigators (several of whom are cited in this paper) that glycogen takes an active part in brain metabolism. Chemical (Kerr & Ghantus, 1937; Khaikina & Gontcharova, 1952; Khaikina, 1957) and histochemical (Sato, 1930–31; Shimizu & Hamuro, 1958) evidence rather strongly supports the concept that there may be more than one fraction of glycogen in the brain.

Beginning with the work of Kerr (1935) and his associates there is general agreement that brain glycogen is relatively stable in the intact animal. However, unless considerable precaution is exercised in extraction procedures, vigorous degradation can be expected. In the present paper nine different procedures were tried with a considerable number of rats before constant, reproducible and adequate values were obtained. A detailed and extensive search of the literature suggests that this is one of the major problems involved with the determination of brain glycogen.

Unpublished results of some experiments from this laboratory indicate that the amount of free glycogen of brain tissue is directly related to the rapidity with which the tissue is frozen. Fasting for 24 hr has no significant effect upon rat brain glycogen, a finding which corroborates another investigation (Lepkovsky, Chari-Bitron & Matzusaki, 1955).

METHODS

A comparison of the glycogen content of the cerebrum, diencephalon and cerebellum was made in fed young and adult white rats of a pure Holtzman strain. Whatever the experimental procedure, all animals were killed by immersion in liquid air to insure uniform and maximum glycostasis (McGinty & Gesell, 1925). Richter & Dawson (1948) suggested that decapitation and placing the head in liquid air was not satisfactory. Palladin (1955) concurs in this. With circulation cut off anoxia occurred and glycolysis continued in the interior of the brain until freezing was complete. The procedure in the present experiments was to place the animal in an oblong, perforated aluminium tube, hinged on the horizontal axis and held by a long wire handle. Complete immersion of the animal and tube in the liquid air was instantaneous. After total freezing the animal was removed and the encephalon sectioned for analysis of the glycogen fractions. The tissue samples were fractionated in the cold room by a method described by Bloom, Lewis, Schumpert & Shen (1951), as modified by Russell & Bloom (1955). The cerebrosides, which reportedly interfere with brain glycogen determination (Winterstein & Hirschberg, 1925), were removed from the tissue residue containing the acid-insoluble or bound glycogen form. This involved washing the residue three times with 2 ml. of a 4:1 mixture of methanol and chloroform. Quantitative estimates of both fractions were carried out by the anthrone method (Seifter, Dayton, Novic & Muntwyler, 1950). All data were subjected to statistical analysis and the null hypothesis rejected at the 0·05 level.

Adult rats. These animals had a weight range from 220 to 260 g and were 4 months of age or older. In addition to a control series, four experimental procedures were performed: (1) an intra-peritoneal injection pentobarbital sodium of 35 mg/kg, (2) an intra-peritoneal injection DNP of 30 mg/kg, (3) experiment 2 in a rat anaesthetized as in 1, and (4) a daily injection DNP of 15 mg/kg for 15 days, followed by anaesthesia and immersion in liquid air. In each of the experiments the animal was killed ½ hr after the final injection.

Young rats. The weight range of these animals was from 90 to 150 g with a maximum age of 55 days. A control plus two experimental groups were analysed. The control series was handled as indicated under adult rats. The experimental series were the same as experiments one and two under adult rats. Experiments involving anaesthesia plus DNP and the daily injection of DNP were not done in these animals.

RESULTS

The results are summarized in Tables 1 and 2. Statistical evaluation of the data in Table 1 indicates that the cerebral hemispheres of the young control animal have significantly less free and total glycogen than the cerebellum and diencephalon, and significantly less bound glycogen than the cerebellum. There are no glycogen differences between the cerebellum and diencephalon. Bound glycogen in the cerebrum, diencephalon and cerebellum amounts to 63·7, 53·1 and 53·1 % of the total glycogen respectively. Anaesthesia in young animals has no effect on the glycogen levels in any of the three areas sampled with the exception that the free glycogen of the cerebrum is increased. No explanation for this increase is readily apparent. Proportionately, bound glycogen is decreased significantly to 42·6 % in the cerebrum and 44·8 % in the diencephalon. A decrease to 49·1 % is observed in the cerebellum but this is not a significant change from the control level. The DNP, as used in these experiments, has no

TABLE 1. Brain glycogen of young rats (mg/100 g)

	No. of animals	Cerebrum			Diencephalon			Cerebellum		
		Bound	Free	Total	Bound	Free	Total	Bound	Free	Total
Control	5	29·0 ± 2·9†	16·5 ± 2·2	45·5 ± 4·8	35·0 ± 1·0	31·0 ± 1·4	66·0 ± 5·1	39·3 ± 2·3	34·7 ± 6·5	74·0 ± 7·6
Anaesthetized	8	25·9 ± 4·1	35·0 ± 4·2	60·9 ± 3·8	29·8 ± 3·1	36·7 ± 3·0	66·5 ± 5·8	35·4 ± 1·7	36·7 ± 5·6	72·1 ± 4·5
Control + DNP*	7	25·9 ± 1·4	13·1 ± 2·1	39·0 ± 3·5	36·4 ± 3·5	24·6 ± 3·2	61·0 ± 5·0	38·5 ± 4·6	25·3 ± 4·3	63·8 ± 7·2

* Killed 30 min after injection. † Standard error of the mean.

TABLE 2. Brain glycogen of adult rats (mg/100 g)

	No. of animals	Cerebrum			Diencephalon			Cerebellum		
		Bound	Free	Total	Bound	Free	Total	Bound	Free	Total
Control	10	45·6 ± 2·7‡	35·2 ± 2·8	80·3 ± 3·7	48·1 ± 2·6	36·0 ± 2·3	84·1 ± 3·8	57·5 ± 2·4	52·6 ± 4·2	110·3 ± 5·3
Anaesthetized	11	32·7 ± 1·3	28·1 ± 2·8	60·8 ± 3·1	36·0 ± 2·0	29·1 ± 4·4	65·1 ± 4·4	33·6 ± 1·3	32·5 ± 2·7	66·1 ± 3·7
Control + DNP*	8	39·0 ± 3·3	19·3 ± 2·5	58·3 ± 4·2	48·1 ± 5·1	23·1 ± 2·2	71·1 ± 5·0	44·3 ± 2·4	27·0 ± 4·4	71·3 ± 6·4
Anaesthetized + DNP*	8	39·7 ± 2·3	27·1 ± 2·2	66·8 ± 2·9	43·7 ± 4·0	30·5 ± 2·2	74·2 ± 4·7	52·3 ± 6·7	37·4 ± 4·1	89·7 ± 9·8
Anaesthetized + DNP†	14	42·7 ± 2·5	28·2 ± 3·1	70·9 ± 4·6	45·5 ± 3·5	28·8 ± 2·3	74·3 ± 4·6	57·0 ± 2·3	38·9 ± 2·6	95·9 ± 3·1

* Killed 30 min after injection. † Daily injection DNP for 15 days. ‡ Standard error of the mean.

effect upon either glycogen fraction in any of the brain areas nor is a significant alteration observed in the ratio of bound glycogen. However, the bound form in the diencephalon in DNP-treated animals is significantly higher than in the cerebrum.

Analysis of Table 2 indicates that the adult cerebral hemispheres and the diencephalon have significantly less bound, free and total glycogen than the cerebellum. There are no differences between the glycogen components of the cerebral and diencephalic areas. Bound glycogen comprises 56·4, 57·5 and 51·8 % of the total in the cerebrum, diencephalon and cerebellum respectively. There is no difference in the amount of bound, free or total glycogen between the three areas sampled after pentobarbital anaesthesia. This suggests that anaesthesia is more effective in lowering these glycogen components in the cerebellum. When compared to the control animals, anaesthesia significantly reduces bound and total glycogen in all areas of the brain and free glycogen in the cerebellum. The ratio of one fraction to the other is not altered. When a control animal is injected with DNP and killed 30 min later, a comparison between the three areas of the brain indicates that they each have approximately the same amount of bound, free and total glycogen. When compared to the control level this metabolic stimulant, however, significantly reduces the free form in each of the three brain regions, the bound component in the cerebellum, and total glycogen in the cerebrum and cerebellum. It significantly increases the proportion of bound glycogen to 66·9 % in the cerebral hemispheres, 67·6 % in the diencephalon, and 62·1 % in the cerebellum. In adult rats anaesthetized and then injected with DNP a significant increase occurs in cerebellar bound and total glycogen over the group subjected to anaesthesia only. The ratio of one fraction to the other is not altered. When the anaesthetized-DNP group is compared to the control-DNP group a significant increase takes place in free glycogen in the cerebrum and diencephalon. Anaesthesia plus DNP also effectively increases the ratio of free glycogen in these two regions of the brain. Anaesthesia may be more effective in maintaining the level of free glycogen in the cerebellum than in the other two areas of the brain.

The fifth group of rats was given a daily injection of DNP for 15 days, anaesthetized and killed. The cerebellum contained significantly more bound, free and total glycogen than did the other two areas but it must be remembered that initially there was more in this phylogenetically older region. When this group is compared to the anaesthetized-only series a significant increase is observed in bound glycogen in all three areas and in total glycogen in the cerebrum and cerebellum. The proportion of one component to the other is changed only in the cerebellum, where the bound form is increased from 50·8 to 59·5 %. A statistical comparison

of the 30-min-anaesthetized-DNP series with the 15-day-anaesthetized-DNP series indicates that there are no significant absolute or proportionate glycogen changes. A significant increase in bound, free and total glycogen is observed in the cerebellum of the 15-day-DNP group in comparison to the control-DNP group. It would appear that here is a second instance where anaesthesia may act to maintain or increase glycogen in the cerebellum. The normal glycogen level in the cerebellum of anaesthetized rats, though significantly reduced from the control level, is maintained in the presence of DNP. Svorad (1959), observing the effect of five different drugs upon glycogen in different areas of the rat brain, reported less variation in the cerebellum than in any of the other regions analysed.

DISCUSSION

The values obtained for normal adult-rat brain glycogen in the present series of experiments are similar to those obtained in earlier investigations (Richter & Dawson, 1948; Palladin, 1955; Khaikina, 1957; Jakoubek & Svorad, 1959; Carter & Stone, 1961). Chesler & Himwich (1943) and Khaikina & Gontcharova (1952) compared brain glycogen in young and adult animals and these data are most closely associated with experiments reported in the present paper (Table 3). Some liberties have been taken with the Chesler–Himwich data, and also with the author's own data for that matter. In order to arrive at a figure representing total whole-brain glycogen it would be necessary to relate glycogen of individual areas of the brain to the weight of these areas. Such information was not available. Consequently, an average value, from those areas analysed by each of the investigators, was derived by simple summation and division. In addition, it has been suggested that killing the animal by intraperitoneal injection of sodium iodoacetate results in a 25 % reduction in brain glycogen (Chance & Yaxley, 1950). This was the method used by Chesler & Himwich, and if their data are increased by this percentage (* in Table 3) a closer correlation for the absolute values of each of the experiments for young animals is realized. Total brain glycogen in young cats, dogs, rabbits and rats is of the same order of magnitude. The polysaccharide is significantly increased in the adult rabbit and rat but not in the adult cat and dog. It was suggested (Chesler & Himwich, 1943) that the concentration of glycogen in the newer phylogenetic areas of the brain increases with age, while that in the older regions decreases with growth. Histochemical studies do not corroborate these results (Shimizu & Kumamoto, 1952). Khaikina & Gontcharova (1952) analysed rabbit brain pulp from embryos, new-born, young and adult animals for bound, free and total glycogen. They concluded that total glycogen was higher in adults than in young animals varying from 2 to 65 days of age. The increase occurred primarily

TABLE 3. Comparison of brain glycogen in young and adult animals (mg/100 g fresh tissue).

	Chesler–Himwich (1943)				Khaikina–Gontcharova (1952) Rabbit						This work Rat					
	Dog Total glycogen		Cat Total glycogen		Young Glycogen			Adult Glycogen			Young Glycogen			Adult Glycogen		
	Young	Adult	Young	Adult	Bound	Free	Total	Bound	Free	Total	Bound	Free	Total	Bound	Free	Total
Cerebral cortex	31	73	45	68	—	—	—	—	—	—	29	17	46	46	35	81
Diencephalon	53	62	30	27	—	—	—	—	—	—	35	31	66	48	36	84
Cerebellum	69	35	97	32	—	—	—	—	—	—	39	35	74	58	53	111
Whole brain	—	—	—	—	30	32	62	61	57	118	—	—	—	—	—	—
Average	51	57	57	42	30	32	62	61	57	118	34	24	58	51	41	92
Plus 25 % of average*	64	71	71	53	—	—	—	—	—	—	—	—	—	—	—	—

* See text for explanation. Note: Chesler–Himwich diencephalon values are represented by the thalamus.

in the bound form. The percentage of free glycogen was greatest in the young groups. Though there may be species differences in the amount of glycogen in young and adult brain tissue, the data obtained from young and adult rat brain are in general agreement with reports suggesting that the adult has a greater concentration.

Chance & Yaxley (1950), on the basis of previous reports from the literature, infer that 'anaesthetization by itself would not appear to be a barrier in the way of obtaining normal glycogen levels'. They suggest, however, that anaesthesia does interfere with central nervous function and therefore it may alter 'the effect of various procedures on the glycogen level of this tissue'. Variable results have been obtained relative to the effects of central nervous system depressants upon brain glycogen (Kerr & Ghantus, 1936; Chance & Yaxley, 1950; Albaum, Noell & Chinn, 1953; Martinson, Tahepold, Hanson, Gurjanova & Hange, 1956; Bronovitskaya & Shapovalova, 1957; Tupikova-Kazimirova, 1957; Isselhard & Thorn, 1959; Svorad, 1959; Carter & Stone, 1961). Species, age, drug, drug concentration, method of glycogen extraction and other parameters probably account for the differences. The data obtained in the present series of experiments on rat brain glycogen indicate one important point relative to the effects of anaesthesia. Brain glycogen of adult rats is significantly decreased by pentobarbital, whereas that of young rats is not affected. A second point is that in the anaesthetized adult the glycogen is not decreased below the normal level of young animals. This does not infer that adult rat brain glycogen cannot be depleted below the level in young animals but it does suggest that the amount of brain glycogen in young animals, as observed in this investigation, is a critical level below which pentobarbital anaesthesia has no effect in either the young or adult rat. This supports the concept that there is tenacious retention of brain glycogen (Kerr & Ghantus, 1936), particularly the bound fraction, at this critical level. There is some doubt as to whether bound glycogen may be converted to the free form, and vice versa, but in the young anaesthetized rat this concept should not be completely excluded. Apparently in the adult rat pentobarbital anaesthesia reduces both fractions at the same rate, but the cerebrum and diencephalon do maintain a greater percentage of bound glycogen as compared to these areas in young anaesthetized rats.

The intraperitoneal injection of DNP was near the LD_{50}. In the adult group of animals this drug is glycogenolytic; in young rats it is not. Analysis of total glycogen only would not reveal this condition but the free fraction was reduced in all three areas of the adult brain. There is also an apparent mobilization of bound glycogen in proportion to the percentage of the free form unless, as has been suggested, there is some conversion between the two components. Experiments *in vitro* with guinea-pig

cortical slices suggest that DNP either inhibits glycogen synthesis (Le Baron, 1955) or stimulates glycolysis (Tsukada, Nagata, Hirano & Takagaki, 1958). Peiss & Field (1948) could not obtain glycogen synthesis in rat cortical slices using a concentration of $1{\cdot}8 \times 10^{-4}$M DNP. The present *in vivo* experiments emphasize the point that brain glycogen in adult animals is more susceptible to the action of pentobarbital and DNP than is that of young rats. In the adult with DNP, as with pentobarbital, the glycogen loss does not go below the normal young level which, as has been mentioned, may be a critical level for encephalic tissue. It is apparent that DNP in an anaesthetized adult rat is not glycogenolytic. After anaesthesia there seems to be some mechanism which prevents glycogenolysis. What sort of a protective or preventative mechanism this may be is not known.

When the drug is given daily for 15 days, as opposed to the single injection, either the anaesthesia is not able to induce the usual glycogenolysis, or the DNP, in some manner, acts to maintain an over-all greater concentration of glycogen. It is also possible that the animal has developed a tolerance to the drug concentration or the dosage may have been of insufficient strength to produce the single-dose response. Free glycogen is reduced to the level of the anaesthetized animal but the more stable form is not changed from the control level.

SUMMARY

1. The adult rat cerebrum, diencephalon and cerebellum have significantly more glycogen than those of the young rat.

2. Pentobarbital anaesthesia significantly reduces bound and total glycogen in all areas of the adult brain but has no effect upon the bound, free or total glycogen in young animals.

3. The drug 2,4-dinitrophenol significantly reduces free glycogen in all areas of the adult brain, bound glycogen in the cerebellum and total glycogen in the cerebrum and cerebellum. It has no effect upon glycogen in the brain of young rats.

4. The glycogenolysis observed in the adult brain with pentobarbital and DNP does not continue below the normal level in young animals. This suggests that the normal amount of glycogen in young rat encephalic tissue may be within a critical range and there is vigorous opposition to degradation.

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INTRACELLULAR RECORDING FROM CELLS OF THE VENTRAL SPINOCEREBELLAR TRACT

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There is an extensive literature on the properties of spinal motoneurones as investigated by the technique of intracellular recording. Especially favourable conditions have been provided by the ease of their identification by antidromic and orthodromic stimulation and by their remarkable tolerance to penetration by the micro-electrodes. Other types of spinal neurones have been much less studied. This paper presents an extensive investigation of the properties of the cells of origin of the ventral spinocerebellar tract (VSCT).

The VSCT neurones which have connexions with afferents in the hind limbs are located in the lumbar segments of the cat spinal cord, the cells of origin extending further caudally than the cells of the dorsal spinocerebellar tract (DSCT) constituting Clarke's column (Oscarsson, 1957*b*). The VSCT cells send their axons across the spinal cord (Oscarsson, 1957*b*; Hubbard & Oscarsson, 1961) to ascend in the superficial region of the ventrolateral and ventral funiculi (see Yoss, 1953).

The properties of the VSCT are known from investigations performed with recording the mass discharge from the whole tract (Oscarsson, 1956, 1960) and with intra-axonal recording from the ascending fibres (Oscarsson, 1957*a*). VSCT neurones are monosynaptically activated by ipsilateral (i.e. contralateral in respect to the ascending fibres) Group I muscle afferents and strongly inhibited by Group II and III muscle afferents and by skin and high-threshold joint afferents (Oscarsson, 1957*a*). Effects of adequate stimulation of receptors have been studied in the spinal (Oscarsson, 1957*a*) and recently in the decerebrate (Oscarsson, 1960) preparation. The transmission to VSCT neurones is subject to control from several supraspinal systems, as has been described in recent papers (Holmqvist, Lundberg & Oscarsson, 1960*a*, *b*; Oscarsson, 1960; Magni & Oscarsson, unpublished).

For the present investigation it is of special interest that all or almost all neurones with contralaterally ascending axons and ipsilateral mono-

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synaptic connexions with Group I afferents reach the cerebellum and can be identified as VSCT neurones (Oscarsson, 1957*a*; Lundberg & Oscarsson, unpublished). We have now recorded intracellularly from more than 60 neurones which were identified as VSCT cells according to the above-mentioned criterion. The location of these cells in the lateral part of the intermediate zone and adjacent parts of the dorsal and ventral horns has been described separately (Hubbard & Oscarsson, 1961).

METHODS

The experiments were performed on cats under light pentobarbitone anaesthesia. In most cases the following left hind limb nerves were dissected and mounted for stimulation: the quadriceps, anterior biceps + semimembranosus, posteror biceps + semitendinosus, triceps surae, plantaris, flexor digitorum longus + flexor hallucis longus, the muscle nerve component of the peroneal (except the nerve to the extensor digitorum brevis), the skin component of the superficial peroneal, and the sural nerve. In several experiments the anterior biceps + semimembranosus and the posterior biceps + semitendinosus nerves were mounted together and called the hamstring nerve. In several experiments the plantaris nerve was mounted on the same stimulating electrodes as the nerves to flexor digitorum longus and flexor hallucis longus.

The spinal cord was exposed by a laminectomy including all lumbar vertebrae, and transected between Th 13 and L 1. The dorsal funiculi were removed for about 2 cm caudal to the transection. In some experiments the spinal cord was then divided into two halves as described by Laporte, Lundberg & Oscarsson (1956) and Oscarsson (1956); in other experiments the left half (ipsilateral to the recorded VSCT cells) was further divided into one ventral and one dorsal quadrant as shown in Fig. 11. In a few cases the right half was similarly divided, permitting stimulation of four separate quadrants of the spinal cord. The dissected halves or quadrants were in continuation with the intact spinal cord at the border between the L 1 and L 2 segments. The dissected parts were mounted on electrodes for stimulation (Fig. 11), one electrode in contact with the severed end, the other on the dissected part close to its site of separation from the intact cord. The ingoing volleys from the stimulated nerves were recorded triphasically from the entrance of the L 6 and L 7 dorsal roots. The descending volleys evoked by stimulation of the dissected parts of the spinal cord were monitored with the same recording electrodes.

VSCT cells were found in the left half of the spinal cord in the upper part of the L 5, and in the lower part of the L 4 segment. The micro-electrode was tilted 5° from the vertical plane, the tip pointing laterally, and pierced the surface of the cord 0·2–0·8 mm medially of the lateral border of the dorsal funiculus. Most VSCT cells were encountered at a depth of 2–3 mm (Hubbard & Oscarsson, 1961). The electrodes were filled with KCl or K_2SO_4 solution. The fixation of the cat on the frame, the micromanipulator and the recording devices have been described previously (Brock, Coombs & Eccles, 1952; Eccles, Fatt, Landgren & Winsbury, 1954; Coombs, Eccles & Fatt, 1955*a*).

Abbreviations

Names of nerves are abbreviated as follows: GR, nerve to gracilis; ST, nerve to sartorius; ABSM, nerve to anterior biceps + semimembranosus; PBST, nerves to posterior biceps + semitendinosus; Q, nerve to quadriceps; GS, nerves to gastrocnemei + soleus; PL, nerve to plantaris; FDHL, nerves to flexor digitorum longus and flexor hallucis longus; PTF, nerves to pretibial flexors (muscle component in peroneal nerve except the nerve to extensor digitorum brevis, the skin components of both superficial and deep peroneal nerves being

removed); SP, skin component of superficial peroneal nerve; S, sural nerve; CP, common peroneal nerve; DP, deep peroneal nerve.

The dorsal and ventral parts of the lateral funiculus (divided at the level of the denticulate ligament) are denoted the dorsolateral and ventrolateral funiculi (DLF, VLF). The VLF + the ventral funiculus is referred to as the ventral quadrant (VQ).

RESULTS

Identification of VSCT cells

The combination of two properties was considered sufficient to identify neurones as VSCT cells: monosynaptically evoked excitatory post-synaptic potentials (EPSPs) from ipsilateral Group I muscle afferents, and antidromic invasion on stimulation of the dissected contralateral spinal half (Hubbard & Oscarsson, 1961). During the investigation it was found that these cells were regularly characterized by other properties. Thus all these cells showed large inhibitory post-synaptic potentials (IPSPs) when skin and high-threshold muscle afferents were stimulated. Excitatory and inhibitory synaptic potentials also appeared when stimulating descending systems according to a characteristic pattern described below (p. 505).

All neurones successfully impaled were tested for antidromic invasion on stimulation of the contralateral spinal half. Of the 103 neurones so responding 65 were identified as VSCT neurones because of monosynaptic EPSPs evoked from Group I muscle afferents. Thirty-two were similar to the VSCT neurones in every respect except monosynaptic excitation. Presumably they received monosynaptic excitation from some of the hind limb nerves not dissected; hence they will be denoted P-VSCT cells (presumed-VSCT cells), and will be so labelled throughout this paper. Only six cells with contralaterally ascending axons had properties different from the VSCT and P-VSCT cells, being distinguished by their connexions both with primary afferents and with descending fibres. They probably do not belong to the VSCT and will not be further discussed. These findings suggest that in the investigated area of the spinal cord the relatively large cells with contralaterally ascending axons are almost all VSCT cells.

External fields and intracellular recording conditions

In a nucleus of motoneurones both antidromic and orthodromic stimulation produce large extracellular fields due to mass activation of the neurones (Brock *et al.* 1952; Eccles *et al.* 1954). These fields can be used for guidance when searching for motoneurones. In contrast, no fields of this type were observed when the micro-electrode was in the region containing VSCT cells. Similarly, external fields were negligible in proximity to Clarke's column (Curtis, Eccles & Lundberg, 1958), which would be expected on account of the very limited zone in which the dendrites of the DSCT cells are confined. No data are available concerning the morphology

of the VSCT neurones. However, the virtual lack of external fields in the region of VSCT cells may be due to the scattering of the cells over a fairly wide area (Hubbard & Oscarsson, 1961). During the search for VSCT cells only the small extracellular fields produced by individual cells could be used for guidance.

On penetration of VSCT cells there was usually an intense injury discharge which often lasted for several minutes. Occasionally the initial

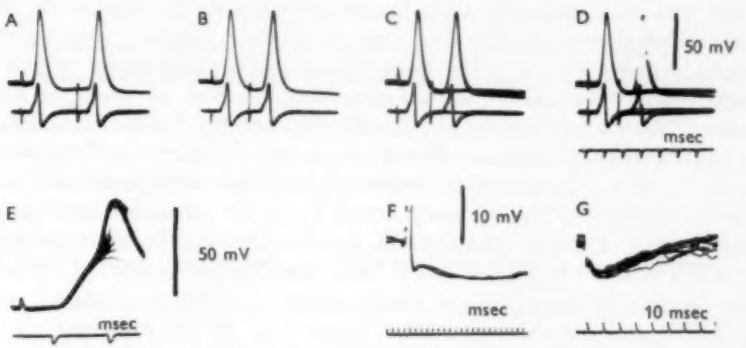

Fig. 1. The action potential in a VSCT cell. This cell had a resting potential of −60 mV and was monosynaptically activated by Ib afferents in the hamstring and gastrocnemius-soleus nerves. All records show antidromic invasion on stimulation of the dissected contralateral spinal half. In A–D two stimuli were applied at decreasing intervals. Upper traces show intracellular potentials, lower the corresponding electrically differentiated records. E was obtained after several seconds of repetitive stimulation at 100/sec. Note occasional block of SD component of spike. F and G were obtained with a slower sweep speed to show after-potentials. Same amplification for F and G. All records obtained by photographic superposition of many faint traces. Note the separate time and potential scales for A–D, for E and for F and G.

resting potential of −40 to −60 mV increased to between −60 and −70 mV and the injury discharge ceased. In some cases this high resting potential was maintained for more than half an hour and permitted investigation of the properties of the spike potential. Usually the resting potential fell below −40 mV with deterioration of the spike mechanism, and blockage of antidromic invasion. Synaptic potentials could then be recorded in isolation, and as this condition of the cell often persisted for a considerable time (more than an hour), extensive investigation of EPSPs and IPSPs was possible. These potentials were little influenced by the subnormal resting potential except for a shortening of their duration which presumably is due to the short time constant of the damaged cell membrane.

The action potential

When a VSCT cell is invaded antidromically by an impulse generated by stimulation of the contralateral spinal half, the spike potential has an inflexion on the rising phase (Fig. 1A–D) suggesting that, as with motoneurones and several other types of nerve cell (Coombs, Curtis & Eccles, 1957; Curtis *et al.* 1958; Eccles, Eccles & Lundberg, 1960), it is compounded of IS and SD spike potentials. However, the IS–SD inflexion is less marked than with motoneurones, though clearly visible in the electrically differentiated traces. Apparently there is a closer coupling between the initial segment (IS) and the soma-dendrite (SD) membranes, which is particularly indicated by the spike potential evoked by a second antidromic volley at an interval of 1–2 msec (Fig. 1C, D). The IS–SD inflexion is accentuated, but there is never a transmission block, i.e. an IS spike in isolation, such as invariably occurs in motoneurones under such conditions (Brock, Coombs & Eccles, 1953). The IS and SD spikes are seen to fail together, which is also observed with high-frequency (250/sec) stimulation of a P-VSCT cell in Fig. 10L. The block may then be occurring between the myelinated axon and the initial segment, as is also observed with motoneurones (Brock *et al.* 1953; Coombs *et al.* 1957). Alternatively, in Fig. 1C, D the second stimulus may have failed to evoke an antidromic impulse in the axon. Occasionally, as in Fig. 1E, blockage between the IS and SD components may develop during prolonged stimulation at high frequencies.

With synaptic activation also there was an IS–SD configuration of the spike though it was less marked than with the antidromic spike. In deteriorated cells the IS and SD components were clearly separated both with synaptically evoked spikes and with spikes spontaneously arising from the slightly fluctuating base line under resting conditions. It is concluded that the IS–SD components exist in the VSCT cells as in motoneurones, but in the closer coupling between these components VSCT cells resemble DSCT cells (Curtis *et al.* 1958; Eccles, Oscarsson & Willis, unpublished observations).

The spike potential declines to an after-hyperpolarization (Fig. 1F, G) which is comparable with that in slightly depolarized motoneurones. There is a brief initial phase which has been attributed to the increased potassium permeability associated with the declining phase of the spike (Eccles, 1957) and a later prolonged phase of about 90 msec (Fig. 1G). Usually the after-hyperpolarization has been between 30 and 70 msec with VSCT and P-VSCT (Fig. 10M) cells, which is thus rather briefer than with motoneurones of limb muscles (Eccles, Eccles & Lundberg, 1958). Presumably the after-hyperpolarization is responsible for the slight depression

of excitability observed for 45–80 msec after antidromic activation of VSCT cells (Oscarsson, 1957*b*, p. 27).

Excitatory potentials evoked by Group I afferents

General features. When latency of the EPSP elicited by Group I afferents is measured relative to the ingoing volley at the dorsal-root entrance 2–3 cm caudally to the VSCT cells, its duration of about 1 msec (Figs. 2, 3, 7 and 9) is accounted for by the synaptic delay plus the conduction time

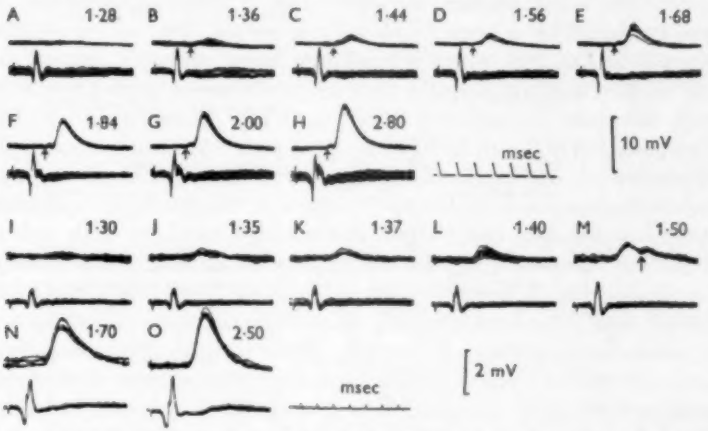

Fig. 2. Monosynaptic EPSPs produced by Ib volley in two VSCT cells, with resting potentials of −30 mV (A–H) and −40 mV (I–O). Upper traces record intracellular potentials, lower traces, the incoming volley at the L7 dorsal root entrance about 3 cm caudal to the VSCT cells. In A–H the posterior biceps + semitendinosus nerve was stimulated with increasing strength. Note brief pre-potential (onset indicated by arrows) representing presynaptic volley in upper traces of records B–H. In I–O the hamstring nerve was stimulated with increasing strength. Note small hump on declining phase of EPSP in record M, which probably represents a single trisynaptic connexion with Group I afferents. All stimulus strengths are indicated relative to threshold strength for that afferent nerve. Every record is formed by superposition of 5–10 traces.

in this 2–3 cm of the primary afferent fibres. When, as in Fig. 2C–H, the monosynaptic EPSP is preceded by a brief wave (indicated by arrows) which is produced by the presynaptic volley in the immediate environment of the VSCT cell, the synaptic delay could be directly measured and was about 0·3 msec. The conduction time in the primary afferents (about 0·7 msec) would correspond to a conduction velocity of 35–40 m/sec for their intraspinal course, which is in reasonable agreement with direct measurements of the velocity in the ascending collaterals of Group I afferents (Lloyd & McIntyre, 1950; Lundberg & Oscarsson, 1956).

Usually the monosynaptic EPSPs of VSCT cells had a duration of only 3–8 msec (Figs. 2, 3, 4), which presumably depends on the brief time constant of the damaged cell membrane. The duration of the normal monosynaptic EPSP has been measured indirectly by testing the decay of the subliminal fringe in a population of VSCT neurones (Oscarsson, 1957*a*, Fig. 6), and found to have a duration (about 15 msec) corresponding to that observed in the same way for motoneurones (Lloyd, 1946); so the EPSPs probably have comparable time courses (see Curtis & Eccles, 1959).

Usually the EPSP declined smoothly (Figs. 2, 3 and 4), indicating that it was produced by a pure monosynaptic action. The sole exception is illustrated in Fig. 2M, where a small late hump probably represents an EPSP elicited through a single trisynaptic connexion with Ib afferents, though Group II participation is not excluded. However, in one VSCT cell and one P-VSCT cell EPSPs appeared with latencies indicating their production by a disynaptic Group I pathway. The VSCT cell was monosynaptically activated by Group I volleys in the nerves to quadriceps, gastrocnemius-soleus, and FDHL, and also activated by a Ib volley in ABSM after a latency 0·65 msec longer than the monosynaptic latency of the same cell (Fig. 7, lower histogram). In the P-VSCT cell a small EPSP appeared with a disynaptic latency on stimulation of the hamstring nerve with a submaximal Group I strength. Since in these three cases hyperpolarizing IPSPs appeared on stimulation of other nerves, these delayed depolarizations could not have been due to IPSPs inverted by chloride diffusion from the electrode (cf. Coombs, Eccles & Fatt, 1955*b*). These late EPSPs were much smaller than the monosynaptic EPSPs and may represent aberrant connexions of little functional significance.

Contribution by Ia and Ib volleys. In almost all cats the afferent volley produced by stimulation of Group I afferent fibres in the hamstring and quadriceps nerves shows a dual contour (Bradley & Eccles, 1953; Eccles, Eccles & Lundberg, 1957*a*). The first component corresponds to Ia fibres from muscle spindles which have a lower electrical threshold and a higher conduction velocity, while the second component corresponds to Ib fibres from Golgi tendon organs (Laporte & Bessou, 1957; Eccles *et al.* 1957*a*; Lundberg & Winsbury, 1960*a*). The mass discharge in the VSCT appears when the stimulus strength is increased to give a Ib volley (Oscarsson, 1956); and isometric contraction of muscles, which stimulates tendon organs, causes excitation of VSCT neurones (Oscarsson, 1960). It has consequently been concluded that the VSCT neurones are activated from Ib but not Ia afferents. This has been confirmed in the present investigation, though exceptions have been noted.

The component of the Group I volley responsible for production of monosynaptic EPSPs was assessed with 44 monosynaptic EPSPs in

37 VSCT neurones. In 41 of these (EPSPs from hamstring or its components in 28, from quadriceps in 4, from gastrocnemius-soleus in 4, from FDHL in 3, and from PTF in 2) the EPSP was evoked by the higher-threshold component of Group I afferents: in the hamstring and quadriceps nerves, by the second component of the ingoing volley; and in other

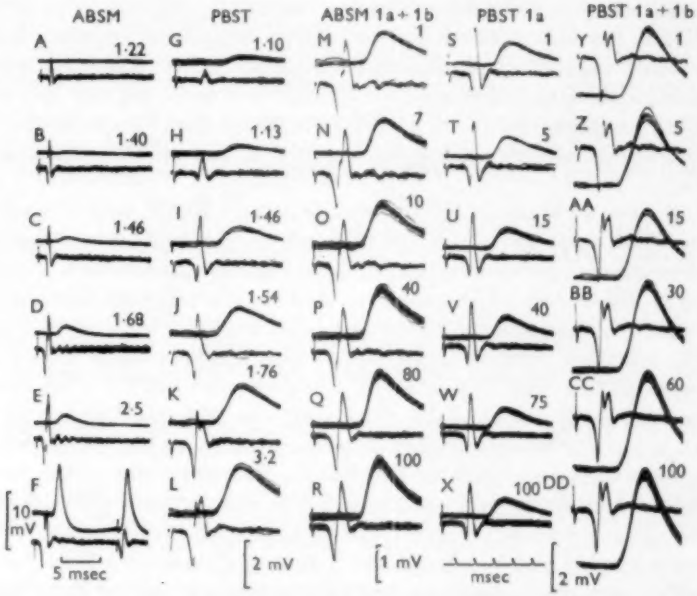

Fig. 3. Convergence of monosynaptic excitation from Ia and Ib afferents on to a VSCT cell (resting potential about −40 mV). Upper traces record intracellular potentials, lower traces, the incoming volley at the L 7 dorsal root entrance 2·6 cm caudal to the cell. Increasing stimulation of the anterior biceps + semimembranosus nerve (ABSM) in records A–E, and of the posterior biceps + semitendinosus nerve (PBST) in records G–L, stimulus strengths being indicated relative to threshold strength. Record F shows the spike responses evoked initially by synaptic stimulation from Group I afferents in the PBST nerve and by antidromic invasion on stimulation of the dissected contralateral spinal half. The spike mechanism deteriorated completely thereafter and there is no trace of it elsewhere in the records of Fig. 3. Records M–DD show variations in size of the EPSP evoked at the various frequencies indicated in cycles per second. The nerves and strengths of stimulation were as follows: M–R, ABSM slightly supramaximal for Group I; S–X, PBST just maximal for Group Ia, but also probably exciting some Ib; Y–DD, PBST slightly supramaximal for Group I. Note that in W–X the ingoing volley is smaller, presumably on account of depressed excitability at such high frequencies. Note also different sweep speeds in records A–F and G–DD. Three different amplifications were used: lower amplification in records A–F, and slightly higher amplification in records M–R, than in records G–L and S–DD.

nerves, by the high-threshold component of the usually single-contoured Group I volley. Two examples are shown in Fig. 2. On successively increasing the stimulus strength the EPSP appears when the Ia volley is maximal or almost maximal and grows with the whole range of the Ib volley. When carefully tested, about ten individual steps of increase could be observed with the EPSP. However, in the upper threshold range of the Ib volley discrete individual steps were especially difficult to discern in the EPSP; hence each EPSP may be built up by action from considerably more than ten individual afferents. The small EPSP, which sometimes appeared with a stimulus strength that seemed to be slightly below Ib threshold, is probably due to Ib afferents which may 'contaminate' the high-threshold part of the Ia volley (Laporte & Bessou, 1957; Eccles *et al.* 1957*a*).

With none of these 41 EPSPs was there any EPSP with a small Ia volley. With three out of the 37 VSCT cells there was good evidence that Ia afferents were partly responsible for the monosynaptic EPSPs. Figure 3 shows a VSCT cell which was activated by Ib afferents from ABSM (records A–E) as shown by the correlation of the EPSP with the Ib volley. On the other hand, an EPSP appeared with a small Ia volley from PBST (G) and was fairly large when the Ia volley was maximal (I). With further increase of the stimulus (J–L), there was a large increase in the EPSP, which approximately parallelled the size of the Ib volley. It is very unlikely that the several steps of EPSP from G to I were due to the few Ib afferents which are sometimes activated with stimulus strengths below the apparent Ib threshold; hence it can be concluded that both Ia and Ib afferents excited this VSCT cell monosynaptically. In two VSCT cells a Ia volley produced monosynaptic EPSPs without any further contribution from the Ib volley in the same nerve. One of the cells was activated by Ib afferents in the quadriceps, ABSM, and FDHL nerves and by Ia afferents alone in the GS nerve. With this cell and the cell of Fig. 3 the characteristic properties of the Ia EPSP at various frequencies of activation have given independent proof that the excitation is from Ia afferents and not from Ib afferents contaminating the Ia volley (see p. 497). In the other VSCT cell quadriceps was the sole nerve which produced excitation; the EPSP appeared with the Ia volley without additional affect from the Ib volley. This cell was otherwise a typical VSCT neurone. We conclude that, in the vast majority of cases, the EPSP in VSCT cells is produced by Ib, but not Ia afferents.

Effect of repetitive activation. The EPSP produced by a Ib volley in VSCT cells characteristically increases in size when the rate of stimulation is increased from 0·5/sec to frequencies as high as 100/sec. In Fig. 4A–F the successive EPSPs exhibit a remarkable potentiation, the increase

being more than 100 % at frequencies of 50–110/sec, but at 350/sec the increase is only about 35 %. In records K–N, from another VSCT cell, the potentiation was smaller, being 25–30 % at 60 and 90/sec, and about 25 % at 140/sec. The potentiation of a second EPSP at a short interval after a previous EPSP is shown in Fig. 4 H–J, where it was 45 % in I and J.

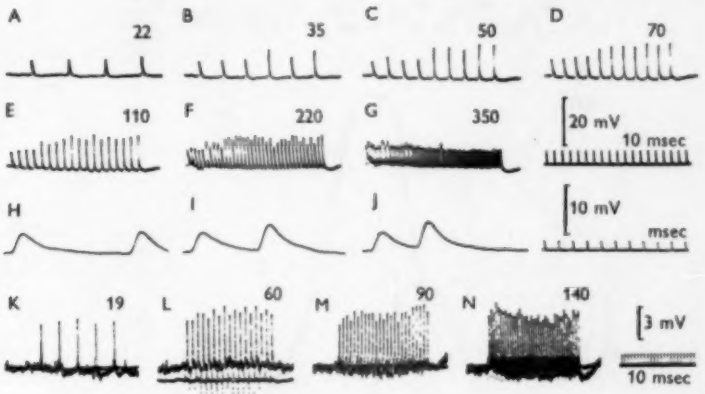

Fig. 4. Effect of repetitive stimulation on size of the Ib EPSP. Records A–G from VSCT cell with a resting potential of −40 mV. Repetitive stimulation of the hamstring nerve at frequencies indicated in cycles per second on records. Note the large increase in size of EPSP with successive stimuli in records C–F. Records H–J from the same cell show increase in size of a second EPSP on a fast time base. Records K–N are from VSCT cell with a resting potential of −30 mV; repetitive stimulation of the PBST nerve at frequencies indicated. The trace with low noise level in K–N signals the afferent volley in the L 7 dorsal root entrance zone, the other is the intracellular record.

Only a few VSCT cells were investigated as in Fig. 4. With most VSCT neurones good recording conditions obtained for only a short time and a more rapid method of testing was adopted. The EPSP was displayed on a fast sweep which was repeated at various frequencies. EPSPs were superposed photographically when a steady state was attained (records in Fig. 5) after the initial 10–20 stimuli (cf. records E–G and L–N in Fig. 4). Since the total duration of the EPSPs was below 8 msec (see above), frequencies up to 125/sec, or even higher, could be used without complication by temporal summation. Typical time courses of the increase of EPSPs with increasing frequency are illustrated in Fig. 5. With Ib activation from the hamstring nerve (Fig. 5) the potentiation of the EPSP was detectable even at 1/sec and reached a maximum at 16–30/sec, but was still considerable at 80/sec. With Ib activation from ABSM nerve (Fig. 5) potentiation of the EPSP was not detectable until the frequency was

10–15/sec and the maximum of about 50 % was attained at about 50/sec. In some cases the maximum was reached at even higher frequencies; for example in Fig. 3Q, R the potentiation increased from 80 to 100/sec, which was the highest frequency tested. Of the 16 Ib EPSPs in VSCT cells

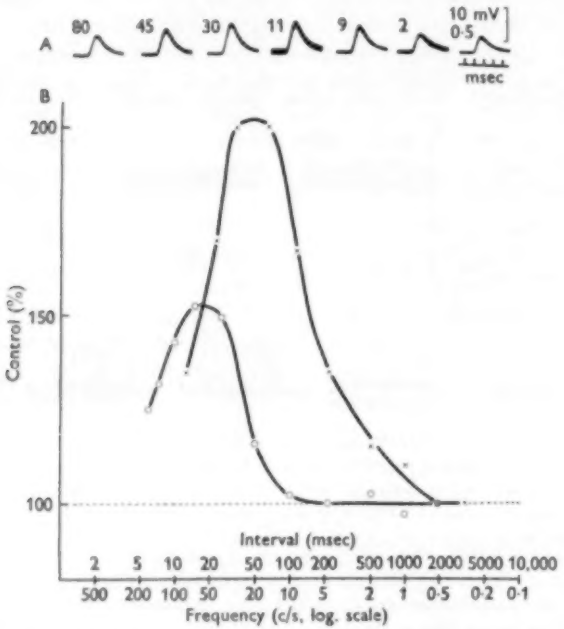

Fig. 5. Change in size of Ib EPSP with variation in frequency. A. The records (5 or more superposed traces) show the EPSPs which maximum Group I hamstring volleys evoked in a VSCT cell with a resting potential of −40 mV, the frequency of stimulation being indicated in cycles per second on the records. Each record was obtained when a steady state of the EPSP size was attained, e.g. after 10–20 stimuli at the high frequencies. B. The curves show the sizes of the EPSPs measured as percentages of that at the lowest frequency (0·3 and 0·5/sec respectively). The upper curve is derived from records partly illustrated in A. The lower curve is from a VSCT cell activated by Ib afferents in the ABSM nerve.

that were so investigated, potentiation started at frequencies between 1 and 5/sec in 5 cases, in the rest between 10 and 20/sec. In no case was there any significant decrease in size (more than 5 %) when the frequency was increased from 0·5 to 30/sec. The maximum potentiation was only 10 % in one VSCT cell, but in 6 it was between 20 and 40 %, and in 9 between 50 and 100%, the optimum frequency usually being at 50–80/sec.

The effect of varying the repetition rate was tested in two of the three VSCT cells activated by Ia afferents. Figure 3S–U shows that the EPSP evoked by the Ia volley decreased in size when the frequency was increased from a low value to 15/sec. With higher frequency the afferent volley decreased (W–X) because of changed excitability of the fibres; hence the

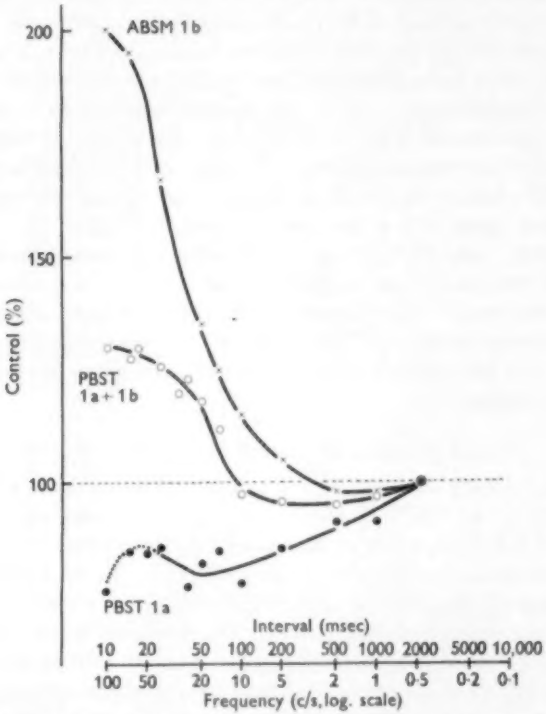

Fig. 6. Sizes of EPSPs produced by Ia and by Ib afferents in a VSCT cell with various frequencies of stimulation. The plotted points are from records partly illustrated in Fig. 3 and are plotted as in Fig. 5. The lower curve is shown by a dotted line over the range of frequencies at which there was some depression of the afferent volley (cf. Fig. 3W–X). Abbreviations as in Fig. 3.

changes in size at these higher frequencies could not be fully assessed. With a slightly supramaximal Group I stimulus to the PBST nerve (Fig. 3Y–DD) potentiation was large at higher frequencies. The plotted curves (Fig. 6) show that the EPSP produced by the Ib ABSM volley started to increase at 5/sec and the size was approximately doubled at 100/sec. On the contrary the Ia EPSP from PBST decreased to about 80% as the frequency increased from 0·5 to 20/sec, and increased again up

to 50/sec, which is comparable with Ia EPSPs in motoneurones (Curtis & Eccles, 1960). At higher rates the ingoing volley decreased and this may be partly responsible for the decline in size shown in the dotted segment of the curve. As would be expected, the EPSP elicited by both Ia and Ib afferents in PBST is a composite of the depressions and potentiations observed for the component Ia and Ib EPSPs. The other VSCT cell was monosynaptically activated by Ib afferents in the quadriceps, ABSM and FDHL nerves and by Ia afferents alone in the GS nerve. The Ib EPSP from FDHL (that from quadriceps and ABSM was not tested) was potentiated by approximately 100%, the increase starting at a frequency of about one per second. The Ia EPSP from the GS nerve resembled the Ia EPSPs of motoneurones (Curtis & Eccles, 1960): a decrease at one per second, with a minimum (80%) at about 5/sec. At higher frequencies the size increased again with a maximum of 95% at 70/sec.

In summary, the Ib IPSP in VSCT cells was potentiated when the stimulation frequency was increased from 0·5 to 30/sec, there being no significant decrease at any frequency. On the other hand, the Ia EPSP in VSCT neurones resembled the Ia EPSP of motoneurones in that it initially decreased, and this decrease was followed by an increase in size at higher frequencies.

Inhibitory potentials evoked by Group I afferents

Inhibitory action from Group I muscle afferents was observed not infrequently in the VSCT neurones. In Fig. 7 the latencies for Group I EPSPs and IPSPs have been measured from the time of the ingoing volley recorded 2–3 cm caudally to the VSCT cells. In the upper histogram the latencies of the EPSPs of 8 VSCT cells (0·9–1·2 msec) indicate a monosynaptic connexion (see p. 491). The latencies of the IPSPs range from 1·7 to 2·1 msec, which gives a mean latency difference between the EPSPs and IPSPs of 0·8 msec. This duration corresponds to the additional delay found on other inhibitory pathways and may be similarly attributed to the interpolation of a single interneurone (cf. Eccles, Fatt & Landgren, 1956; Eccles & Lundberg, 1958*a*; Curtis *et al.* 1958; Araki, Eccles & Ito, 1960; Eide, Lundberg & Voorhoeve, 1960). Comparable latency differences are shown in the lower histogram, representing data from an experiment in which 10 VSCT and 7 P-VSCT cells were studied. The IPSPs in this diagram are grouped around two maxima which probably represent disynaptic and trisynaptic connexions. It should be observed that the latency difference between the two groups of IPSPs is no more than 0·6 msec, which is close to the minimal value observed for interpolation of an interneurone. Every IPSP of VSCT cells has exhibited the additional latency, relative to the monosynaptic EPSP, that is illustrated

in Fig. 7, so it may be concluded that the inhibitory pathways to VSCT cells resemble those to motoneurones and DSCT neurones in having at least one interpolated interneurone.

In a few experiments with recording from axons of VSCT neurones, testing with increasing strength of stimulation showed that the inhibition appeared with the Ib volley (Oscarsson, 1957*a*). With the more sensitive

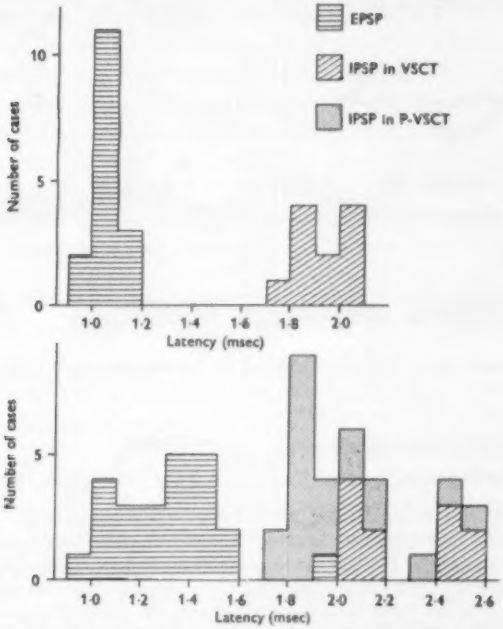

Fig. 7. Comparison of latencies of EPSPs and IPSPs evoked by Group I afferents. Histograms show distribution of latencies in two experiments. The upper histogram is plotted from an experiment in which 8 VSCT cells were encountered; the lower, from an experiment in which 10 VSCT and 7 P-VSCT cells were encountered. The IPSPs produced in P-VSCT cells are marked by stippling. The latencies were measured from the incoming volley recorded 2–3 cm caudal to the cells.

method of intracellular recording from VSCT cells it has been shown that inhibition is frequently produced by both Ia and Ib afferents. The Group I component responsible for the IPSP was assessed in a number of cases showing good separation of Ia and Ib volleys in the quadriceps and hamstring nerves. In Fig. 8A a small Group Ia quadriceps volley produced a disynaptic IPSP which increased when the strength was increased to give a maximal Ia volley in B. The increased and prolonged IPSP when the

strength was raised from 2·1 to 3·0 times threshold (D–F) suggests inhibition from Group II afferents, while Group III afferents were probably responsible for the later IPSP observed at 12 times threshold in J. Group I inhibition with the long latency characteristic of polysynaptic connexions was sometimes observed. For example, in Fig. 8K an almost maximal

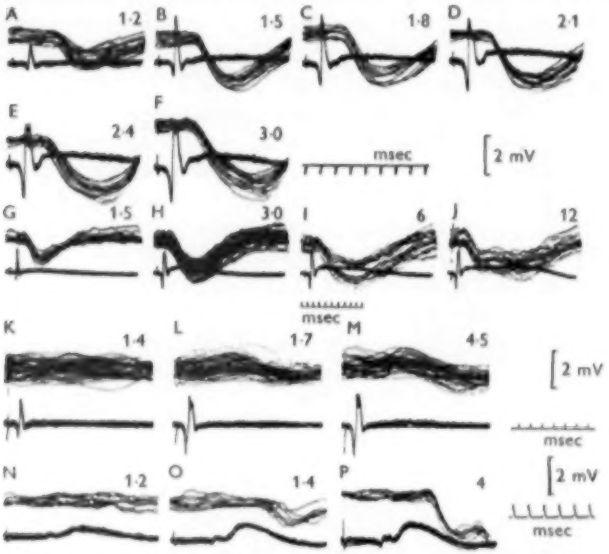

Fig. 8. Inhibitory potentials evoked in VSCT cells. Upper traces record intracellular potentials, lower traces the incoming volley at the L 7 dorsal root entrance about 3 cm caudal to the cells. A–J, IPSPs produced by Q volleys at increasing strength of stimulation in a VSCT cell (resting potential, −45 mV) that was monosynaptically excited from the PBST, ABSM, and G8 nerves. K–M are records from a VSCT cell (resting potential, −40 mV) that was monosynaptically activated from the PBST nerve, and show effect of increasing strengths of stimulation of the ABSM nerve. Records N–P are from a VSCT cell (resting potential, −50 mV) that was monosynaptically activated from the PBST, ABSM, and G8 nerves and show effect of increasing strengths of stimulation of the cutaneous component of the superficial peroneal nerve (SP). All stimulus strengths are indicated relative to the respective threshold strengths. Abbreviations p. 487.

Group Ia volley from ABSM nerve produced no inhibition, while in Fig. 8L a small EPSP and later IPSP was evoked by a volley almost maximal for Ib as well as Ia. Additional activation of Group II afferents (Fig. 8M) only produced a slight increase in the EPSP and prolongation of the IPSP.

Ia inhibition with disynaptic latency, as in Fig. 8A, was found in six of the eight VSCT cells and in both the P-VSCT cells in which IPSPs were

produced by quadriceps afferent volleys; there was Ib inhibition in the remaining two VSCT cells. There was also Ib inhibition in the three cells in which hamstring Group I volleys gave IPSPs. With the Ib IPSPs the latency varied between 2·3 and 4 msec, suggesting that the pathway included two or more interneurones. In most of the cells with Ia IPSPs, there was evidence of an additional Ib inhibition (Fig. 8A–J). With several other VSCT cells GS and FDHL volleys evoked by weak stimuli produced IPSPs with a latency suggesting disynaptic linkage, while higher threshold Group I afferents produced a prolongation of the IPSP.

In summary: VSCT neurones were inhibited from Ia and Ib afferents. The Ia inhibition was disynaptic while the Ib inhibition was, largely at least, tri- or polysynaptic. This organization is not unlike the organization of Group I inhibition to motoneurones, where there is disynaptic inhibition from Ia afferents (Eccles *et al.* 1956) and di- and trisynaptic inhibition from Ib afferents (Eccles *et al.* 1957*c*). With Ib inhibition in VSCT neurones the trisynaptic linkage dominates, but some of our records actually suggested that Ib afferents sometimes have a disynaptic linkage.

Synaptic actions evoked by Group II and III muscle afferents and by skin afferents

Group II and III volleys from all tested muscle nerves evoked inhibition in the VSCT and P-VSCT cells. Often the inhibition from Group II was fairly weak or even absent, but large IPSPs were then produced by Group III afferents. Differences in the contribution to inhibition from Group II and III afferents may represent differences in the general condition of the preparation rather than intrinsic differences in synaptic coupling (cf. Eccles & Lundberg, 1959). Similarly, cutaneous afferents always produced inhibition. Sometimes the low-threshold afferents were effective, as in Fig. 8N–P, but sometimes stimulation of 5–10 times threshold strength was needed for an effect. Cutaneous and Group II muscle afferent volleys rarely had a synaptic excitatory action on VSCT cells. When present, the EPSPs were small (Figs. 8L, M; 9J, K) and had a latency suggesting a di- or polysynaptic pathway.

Previously VSCT units have been divided into I- and E-types (Oscarsson, 1957*b*), but in the present investigation the patterns uniformly conformed to the I-type, which is characterized by inhibitory action from high-threshold muscle and skin afferents. The E-units, which constituted more than 10% of the total number of previously investigated VSCT units, had a similar organization but received in addition an early phase of excitation from these afferents. Presumably these latter neurones are located at a different segmental level and have escaped recording during the present investigation. The small initial EPSP sometimes observed in the present

experiments (e.g. Fig. 8L, M) may correspond to the excitatory action which, in the E-units, is much more pronounced.

Patterns of convergence

Monosynaptic excitation from Group I afferents is limited to some ipsilateral muscle nerves (Oscarsson, 1957*b*) and disynaptic Group I

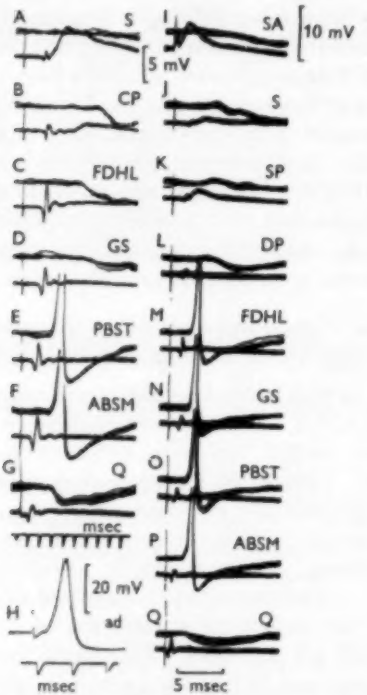

Fig. 9. Patterns of excitatory and inhibitory convergence on two VSCT cells (resting potentials of about −60 mV). Lower traces in A–G and I–Q record the incoming volley at the L7 dorsal root entrance about 2·5 cm caudal to the cells. Records A–H are from one cell, H showing the antidromic spike on stimulation of the contralateral dissected spinal half. Records I–Q are from another VSCT cell. Antidromic invasion was observed, but the cell was lost before records had been taken. The various nerves which were stimulated are indicated on each record (abbreviations p. 487; SA = sacral roots 2 and 3). The stimulus strength was supramaximal for Group I but not for Group II afferents.

inhibition is even more restricted. Synaptic actions in two typical VSCT neurones are shown in Fig. 9. In A–G there is monosynaptic excitation from the two hamstring nerves and disynaptic inhibition from the quad-

riceps nerve, while all other nerves produced late inhibition due to cutaneous and high threshold muscle afferents. The stimulus strength was adjusted to excite the majority of Group II but not Group III afferents. Possibly traces of inhibition can be seen superposed on the hyperpolarization following the spike in records E and F, but a large late IPSP was produced with Group III stimulus strength (not shown), which is the usual result for muscle nerves supplying monosynaptic excitation. More extensive excitatory convergence is shown in Fig. 9 I–Q from another VSCT cell. EPSPs are elicited from the hamstring nerves and also from the triceps and FDHL nerves, while the quadriceps nerve again supplied disynaptic inhibition.

TABLE 1. Convergence of monosynaptic Group I excitation (EPSP) and disynaptic Group I inhibition (IPSP) from various nerves (abbreviations on p. 487) on to 15 VSCT neurones monosynaptically activated from the hamstring but not the quadriceps nerve

	Q	PBST + ABSM	GS	FDHL + PL	PTF
EPSP	0	15	10	8	1
IPSP	14	0	1	2	1
Nil	1	0	4	5	13

TABLE 2. Convergence of monosynaptic Group I excitation (EPSP) and disynaptic Group I inhibition (IPSP) from various nerves (abbreviations on p. 487) on to 19 VSCT neurones monosynaptically activated from the quadriceps nerve. In 5 cells the quadriceps Group I volley produced both EPSPs and IPSPs. Figures within brackets refer to convergence of Group I effects on to the subgroup of VSCT neurones activated from both the quadriceps and FDHL + PL nerves

	Q	ABSM	PBST	GS	FDHL + PL	PTF
EPSP	19 (9)	8 (5)	0 (0)	7 (7)	9	2 (1)
IPSP	5 (2)	2 (1)	1 (0)	3 (0)	3	3 (2)
Nil	0 (0)	9 (3)	18 (9)	9 (2)	7	14 (6)

Tri- and polysynaptic inhibition by Group Ib afferent volleys occurred from a more extensive receptive field than the disynaptic IPSP, but usually has not been distinguished from Group II and III inhibition in our experiments.

In our experiments 37 VSCT neurones were tested for monosynaptic excitation and disynaptic inhibition from the nerves listed in Tables 1 and 2. Table 1 gives the convergence patterns for the 15 neurones activated from the hamstring but not the quadriceps nerve. It appears that the majority of these neurones are also monosynaptically excited by both the GS and FDHL + PL nerves, whereas all except one are disynaptically inhibited from the quadriceps nerve. These results are similar to those obtained previously, though in our present experiments inhibition from the quadriceps nerve was more frequent (Oscarsson, 1957*b*). Table 2 shows convergence of Group I effects on 19 VSCT cells monosynaptically activated from the quadriceps nerve. There was never any excitation from the

antagonist to the quadriceps, the knee flexor PBST, and only in one cell did it produce an IPSP. Six cells were activated from quadriceps alone, and with the others there was considerable convergence from the nerves to extensor muscles at the various joints; hip (ABSM), ankle (GS) and toe joints (FDHL + PL). The FDHL + PL nerve excited 9 cells which seem to form a subgroup among those activated from the quadriceps nerve. Figures within brackets in Table 2 represent the convergence on these cells; not less than 7 were in addition activated from the gastrocnemius-soleus nerve and five from the ABSM nerve. The results reported previously (Oscarsson, 1957*b*) differ mainly in respect of the occasional occurrence of excitation from the PBST nerve.

Three of the VSCT neurones are not included in Tables 1 and 2. Two were activated from the PTF nerve only and the remaining one from both the GS and FDHL + PL nerves.

Convergence of monosynaptic excitation from nerves to antagonist muscles was very uncommon: the quadriceps and PBST nerves were never observed to excite the same VSCT cell; only one of the 5 VSCT cells excited by PTF was also excited from the antagonist muscles, GS and FDHL + PL.

Disynaptic inhibition occurred most often from the quadriceps nerve; that is, in all but 1 of the 15 VSCT cells activated from the hamstring nerve; in 5 of the 19 cells activated from the quadriceps nerve; and in 1 of the 2 cells only activated from PTF. Otherwise inhibition was uncommon even from nerves to antagonists of the muscles supplying excitation. Only one of the VSCT cells monosynaptically activated by quadriceps was disynaptically inhibited from PBST; there was disynaptic inhibition from the PTF nerve in 3 of the 20 cells activated from the GS and/or FDHL + PL nerves. Both di- and polysynaptic Group I inhibition from the PBST nerve were uncommon in neurones activated from the quadriceps nerve.

The convergence of synaptic action from various nerves on to a typical P-VSCT cell is illustrated in Fig. 10. Inhibitory action appeared with Group I strength on stimulation of the quadriceps, GS, and the FDHL nerves. The latency with these three nerves indicated a disynaptic linkage, as is shown for the GS and FDHL nerve on a fast sweep (records I and J), though there was in addition a polysynaptic pathway. The other nerves produced inhibition from skin and high-threshold muscle afferents after a relatively long latency. Records K and L show the antidromic spike, K at a frequency of 10/sec and L at 250/sec. As with typical VSCT cells there was no separate block of the SD spike; at the higher frequency there was sometimes a complete block of invasion (L). Record M shows the after-hyperpolarization which had a duration of approximately 50 msec.

Of our total of 32 P-VSCT cells 24 received disynaptic inhibition from one or several nerves while 9 had only late inhibition. The disynaptic

inhibition followed a similar pattern to that of VSCT cells, being common from the quadriceps nerve and very uncommon from the hamstring nerve.

Synaptic potentials evoked by stimulation of the cord

Several descending supraspinal control systems influence the VSCT cells. It has recently been shown that fibres in the ipsilateral dorso-lateral

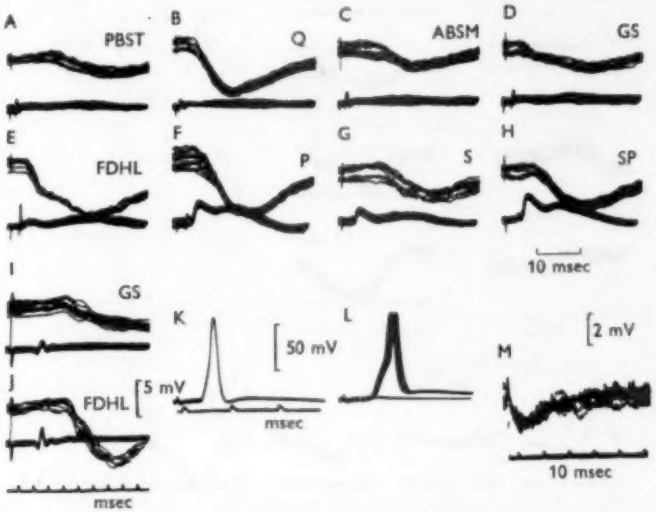

Fig. 10. Convergence on a P-VSCT cell. Upper traces record intracellular potentials, lower traces, the incoming volley at the L7 dorsal root entrance. Resting potential −50 mV when records A–J were obtained, and −76 mV when records K–M were obtained. Records A–H: stimulation of the various nerves indicated by abbreviations (p. 487). The stimulus strength was supramaximal for Group II afferents. Records I and J show on faster sweep the potentials elicited from GS and FDHL nerves. Records K and L show the antidromic spike appearing on stimulation of the contralateral dissected spinal half. K at a frequency of 10, L at 250 stimuli per second. The after-hyperpolarization following the spike potential is shown in record M. Note separate time scales for A–H, I–J, K–L, and M.

funicle belonging to the pyramidal tract have inhibitory connexions with VSCT cells (Oscarsson, 1960; Magni and Oscarsson, 1961). Other fibres with high conduction velocity descend in the ipsilateralventral quadrant of the cord and exert monosynaptic excitatory action on the VSCT cells (Oscarsson, 1957*b*, p. 68). These latter fibres probably originate from cells in the brain stem and convey effects from the cerebellar cortex (Holmqvist *et al.* 1960*b*). Both VSCT and P-VSCT cells exhibited synaptic potentials presumably generated by connexions with these two descending systems.

Typical effects on stimulation of the various dissected bundles of the spinal cord (diagram of Fig. 11) are shown in Fig. 11A–G, the distance between the stimulating electrodes and the VSCT cell being 3·6 cm.

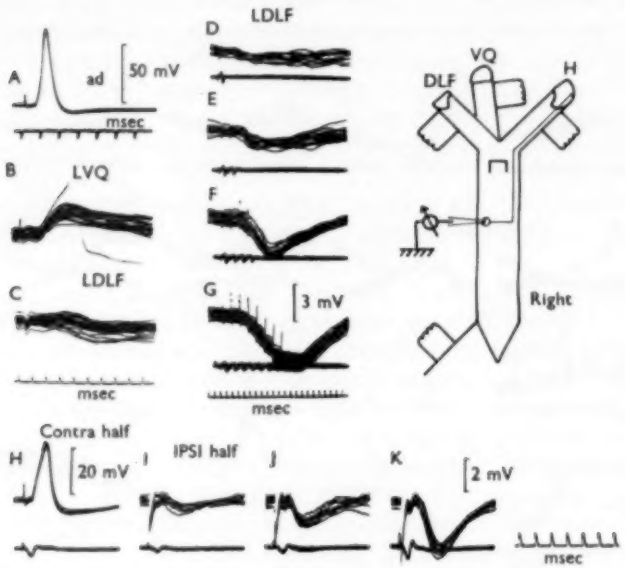

Fig. 11. Synaptic potentials evoked by stimulation of tracts in the spinal cord. Records A–G are from a VSCT cell (resting potential −70 mV) activated by the GS, FDHL, and plantaris nerves. Diagram of recording and stimulating arrangement shown to the right. A, stimulation of the contralateral spinal half (except dorsal funiculus) results in antidromic invasion (ad). B, stimulation of the ipsilateral ventral quadrant (LVQ). C, stimulation of the ipsilateral dorsolateral funiculus (LDLF). Records D–G as for C but with slower sweep speed and increasing number of stimuli. Distance between the VSCT cell (upper part of L5 segment) and stimulating electrodes on the dissected bundles of the cord was 3·6 cm. Records H–K are from a P-VSCT cell, resting potential −45 mV. In this experiment only the two spinal halves (except dorsal funiculi) were dissected. H, antidromic invasion from contralateral spinal half. I–K show an IPSP appearing on stimulation with increasing strength of the ipsilateral half. Note short latency, the conduction distance being 2·1 cm. The lower traces in D–G and H–K record the descending volley at the L7 segment.

Record A shows the antidromic spike elicited by stimulation of the contralateral spinal half (except the dorsal funicle). Stimulation of the ipsilateral ventral quadrant evoked an EPSP with a latency of 1·25 msec (B). Stimulation of the ipsilateral dorsolateral funicle gave an IPSP with a latency of about 3 msec (C), which was strongly potentiated by repetitive

stimulation, as illustrated in records D–G. These respective EPSPs and IPSPs are characteristic of both VSCT and P-VSCT cells. Only very occasionally were there additional responses—a small EPSP on stimulation of the dorsolateral funicle, or a small IPSP on stimulation of the ventral quadrant. These occasional findings are probably due to a dissection of the cord funiculi which did not completely separate the

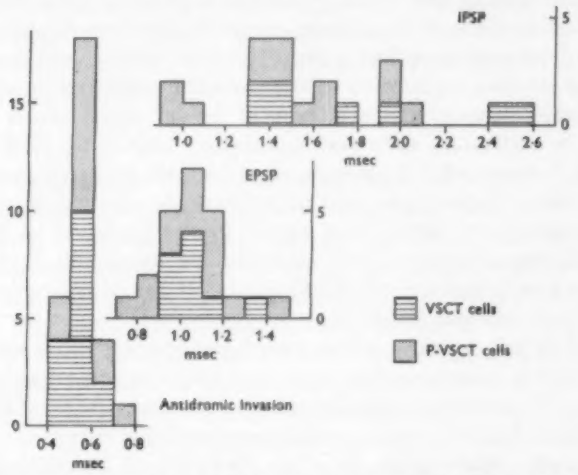

Fig. 12. Latencies of antidromic spike and synaptic potentials evoked by stimulation of dissected spinal funiculi (cf. diagram in Fig. 11), from three experiments with distances between VSCT cells and stimulating electrodes of 2·1–3·0 cm. Antidromic invasion was obtained by stimulation of the contralateral dissected spinal half (except dorsal funiculus). In one of the experiments the ipsilateral half (except dorsal funiculus) was dissected, in the other two this half was divided into ventral and dorsal quadrants, as in the diagram of Fig. 11. EPSPs appeared on stimulation of the ipsilateral half or the ipsilateral ventral quadrant, except for two cells in which EPSPs also appeared on stimulation of the ipsilateral dorsal quadrant. IPSPs appeared on stimulation of the ipsilateral half or ipsilateral dorsal quadrant, except for one unit in which an IPSP appeared also on stimulation of the ipsilateral ventral quadrant. The abscissa indicates the latency in msec. The ordinate indicates the number of cases. Both VSCT (crosshatched) and P-VSCT (stippled) cells are represented.

tracts responsible for the respective effects. In a few experiments also the contralateral half was divided into dorsal and ventral parts. No synaptic potentials appeared on stimulation of the dissected contralateral dorsolateral funicle, while, as expected, stimulation of the ventral quadrant resulted in antidromic invasion of the cells.

Figure 12 shows the distribution of latencies of the various responses evoked by stimulation of the spinal cord in three experiments in which the

distance between the stimulating electrodes and the recorded VSCT and P-VSCT cells varied between 2·1 and 3·0 cm. Previous investigations have indicated that the three fibre systems have high and fairly similar conduction velocities—usually between 80 and 120 m/sec for VSCT fibres (Oscarsson, 1957*b*), approximately 100 m/sec for the fastest fibres in the system descending in the ventral quadrant (Holmqvist *et al.* 1960*b*), and about 85 m/sec for the fastest fibres producing inhibition (Oscarsson, 1960). The conduction distance is the same, except for the short distance added to the VSCT fibres because of the traverse of the cord; hence the minimal latency differences between the various effects are mainly due to synaptic delays.

Antidromic invasion had a latency of 0·4–0·7 msec, which would be occupied in utilization time and conduction time. The EPSP had an additional latency of 0·3 msec or more, which is attributable to the synaptic delay. Similar durations for synaptic delays have been observed with monosynaptic activation of motoneurones (Brock *et al.* 1952) and with Ib afferent volleys on VSCT neurones (see above, Fig. 2). The longer delay found with some of the EPSPs may be due to a slow conduction velocity in the afferent fibres.

The IPSPs had latencies which varied considerably (Fig. 12). A peak around 1·4–1·6 msec indicates that the connexion in most cases was disynaptic. The latency difference between the main group of IPSPs and the EPSPs is about 0·6 msec, a value approximating that found for interpolation of an interneurone in other pathways of the nervous system (Eccles *et al.* 1956; Eccles *et al.* 1957*c*). The latency of the IPSPs was in many cases so long that the interpolation of two or more interneurones must be presumed. Even longer latencies than those shown in the histogram sometimes occurred in other experiments (Fig. 11).

It is of special interest that the inhibition had a monosynaptic latency in three cells (Fig. 12). For example, Fig. 11H shows the antidromic spike appearing on stimulation of the contralateral half, and records I–K show the IPSP appearing on stimulation of the ipsilateral half. The IPSP appears with a weak stimulus as indicated by the small descending volley recorded at the L7 dorsal root level (I) and grows with increasing strength (J, K). Its appearance at a weak stimulus strength excludes the possibility of stimulus escape to a more caudal region of the cord nearer to the VSCT cells. There is convincing evidence that in the central nervous system synaptic inhibitory action is exerted by special interneurones which have been called inhibitory cells. These inhibitory cells in the intermediate nucleus have been directly stimulated by Eide *et al.* (1960), and a comparable direct stimulation of inhibitory cells must be postulated when the IPSP has the very brief latency shown in Fig. 11I–K. Such inhibitory cells must have axons at least 2 cm long.

The IPSP always increased in size with a train of stimuli, as in records D–G, Fig. 11. In some cases no potential appeared with a single stimulus, but a large IPSP was then evoked by two or more stimuli. The increase in inhibition with successive stimuli may be due to several factors. One possibility is that the EPSPs generated in the inhibitory interneurones by the stimulated fibres grow with successive activation, just as was shown for the EPSPs produced by Ib afferents in VSCT neurones. Hern, Landgren & Phillips (1961) have indeed recently shown that EPSPs produced by pyramidal-tract fibres in motoneurones of the monkey increase in size severalfold during repetitive stimulation.

DISCUSSION

The present investigation is based on identification of VSCT neurones by the combination of two properties: monosynaptic activation from ipsilateral Group I muscle afferents and antidromic invasion from contralaterally ascending axons. Previous investigations showed that the combination of these two properties occurs only with VSCT cells (Oscarsson 1957*b*, p. 12; Hubbard & Oscarsson, 1961; Lundberg and Oscarsson, unpublished). VSCT neurones have several other characteristic features. (i) In the spike potential IS and SD components differ from motoneurones in showing the same close coupling that occurs with DSCT neurones (Eccles, Oscarsson & Willis, unpublished observations). (ii) Large and prolonged IPSPs are regularly produced by cutaneous volleys and by Group II and III volleys from all the various hind-limb muscles. (iii) The VSCT cells are inhibited on stimulation of the ipsilateral dorsolateral funicle, which presumably is due to stimulation of the pyramidal tract (Oscarsson, 1960; Magni & Oscarsson, unpublished observations). (iv) Monosynaptic EPSPs are regularly produced by stimulation of the ipsilateral ventral quadrant, which is probably due to a system of descending fibres mediating effects from the cerebellum (Oscarsson, 1957*b*, p. 68; Holmqvist *et al.* 1960*b*). (v) Finally, the VSCT neurones are located in the lateral part of the intermediate zone and adjacent parts of the dorsal and ventral horns (Hubbard & Oscarsson, 1961).

Previous investigations indicated that VSCT neurones are monosynaptically excited by Golgi tendon-organ afferents but not by Group I muscle-spindle afferents: the mass discharge in the VSCT appeared with stimulation of Group Ib afferents (Oscarsson, 1956); and isometric contraction of muscle, which strongly activates tendon organs, evoked excitation in VSCT neurones (Oscarsson, 1960). In the present investigation 44 monosynaptic EPSPs in VSCT neurones were studied; the EPSP was evoked by the Ib volley in 41 cases, while in one it appeared with both Ia and Ib volleys, and in two with the Ia volley alone. A few Ib afferents sometimes

'contaminate' the Ia volley (Laporte & Bessou, 1957; Eccles *et al.* 1957*a*), but it seems unlikely that these could account for these three instances of apparent Ia EPSP. First, many individual steps of the Ia EPSP could be distinguished by threshold discrimination, which shows that there were a considerable number of effective afferents within the Ia range. Secondly, with two of these VSCT cells the EPSP was tested by varying the stimulation frequency. While the EPSPs induced by the Ib volley characteristically increased in size on increasing frequency, the Ia-induced EPSPs decreased in size, just as the Ia EPSPs in motoneurones (Curtis & Eccles, 1960). It may be concluded that the vast majority of VSCT neurones are monosynaptically activated only by Ib afferents, but a few receive monosynaptic excitation from Ia afferents.

Two of the VSCT cells which were monosynaptically excited by Ia afferents had monosynaptic connexions with Ib afferents in other nerves. Hitherto, convergence of monosynaptic excitation from Ia and Ib afferents has never been established unambiguously. With motoneurones such convergence does not occur, for there is never monosynaptic activation from Ib afferents (Eccles *et al.* 1957*a*, *c*), and the interneurones in the intermediate zone seem to be excited either by Ia or Ib afferents (Eccles *et al.* 1960). These and similar findings (cf. Eccles & Oscarsson, 1961) suggest that, as a general rule, neurones do not receive monosynaptic excitation from more than one type of primary afferent. Though this rule may hold generally, there are clear exceptions among the VSCT neurones. With DSCT neurones convergence of Ia and Ib excitation was originally suggested by Lundberg & Oscarsson (1956) to occur occasionally, but experiments with adequate stimulation of receptors (Lundberg & Oscarsson, 1956; Lundberg & Winsbury, 1960*b*) indicated that the DSCT neurones function as a pathway either for muscle spindles or for Golgi tendon organs. Recent intracellular records from DSCT neurones have, however, disclosed that Ia and Ib afferents sometimes converge on to the same DSCT cell (Eccles *et al.* 1961).

Presumably the occasional convergence of the two types of Group I afferents should be regarded as a kind of aberrant connexion without much functional significance. This is certainly likely with DSCT neurones (Lundberg & Oscarsson, 1956; Lundberg & Winsbury, 1960*b*) and is probably also true with VSCT cells. In the present investigation the three cases of Ia activation occurred from nerves to three different muscles, two extensors and one flexor. This excludes the possibility that the Ia connexion is related to any certain muscle group with special function. Furthermore, there is evidence that the VSCT neurones in both a marsupial—the Australian possum—and the rabbit receive monosynaptic excitation from Ib but not Ia afferents (Magni and Oscarsson, unpublished observations).

When the VSCT neurones in the cat receive Ib but not Ia activation, they evidently conform to a phylogenetically old pattern of organization.

Curtis & Eccles (1960) showed that the Ia EPSP in motoneurones characteristically changed in size when the repetition rate was altered. With increasing frequency the size of the EPSP invariably decreased to reach a minimum at frequencies between 4 and 20/sec. At higher frequencies there was a partial recovery, or even an increase above the control value. On the other hand, the Ib EPSP in VSCT cells never decreased significantly in size and increased at higher frequencies to between 110–200 % of the control value. It has been suggested (Curtis & Eccles, 1960) that the change in size of the Ia EPSP in motoneurones is due to the interaction of two processes, depletion of the transmitter substance which dominates at low frequencies and mobilization of the transmitter substance which counteracts the depletion at higher frequencies. The difference between the behaviour of the Ia EPSP in motoneurones and the Ib EPSP in VSCT neurones would be explained if the depletion process in the Ib afferents is insignificant, possibly because of a large reserve of transmitter substance. The range of frequencies giving optimal increase in size was similar for Ia and Ib EPSPs. The increase was, however, much more marked with the Ib EPSPs, which suggests a more effective mobilization process. The potentiation of EPSPs with increasing frequency is not a property uniquely correlated with the Ib afferents; not only does it exist with Ia afferents, but it seems to be even better developed with monosynaptic connexions of pyramidal tract fibres on motoneurones (Hern *et al.* 1961).

Differences between the monosynaptic Group I connexion to motoneurones on the one hand, and to VSCT and DSCT neurones on the other hand, are revealed by the fact that motoneurones only follow low frequencies of presynaptic stimulation (Adrian & Bronk, 1929; Lloyd, 1957), while the tract neurones may follow frequencies of several hundreds per second (Holmqvist, Lundberg & Oscarsson, 1956; Oscarsson, 1957*b*). With motoneurones it is likely that the after-hyperpolarization (Eccles *et al.* 1958) and the recurrent inhibition through Renshaw cells (Holmgren & Merton, 1954; Granit, Pascoe & Steg 1957; Kuno, 1959; J. C. Eccles, R. M. Eccles, A. Iggo and M. Ito, unpublished observations) limit their ability to discharge at high frequencies. Though VSCT and DSCT neurones have an after-hyperpolarization, which is comparable with that of motoneurones, it causes only a slight depression of their excitability (Oscarsson, 1957*b*, p. 27), and there is no evidence that the tract neurones receive recurrent inhibition. Another factor adapting VSCT cells to high-frequency discharge is the increase in size of the Ib EPSP with increasing frequency. A further factor of importance is the size of the EPSP in relation to the threshold for

initiation of axonal spikes. This factor can rarely be assessed with VSCT neurones because the low resting potential makes the recording conditions too unfavourable. It is, however, probably of importance for transmission to the DSCT neurones, which have large EPSPs, which are presumably produced by the giant synapses described by Szentagothai & Albert (1955).

The ability of the tract neurones to follow high repetition rates for a long time (cf. Oscarsson, 1957*b*) may possibly be related to a characteristic feature of the post-tetanic potentiation in the tracts. In the monosynaptic reflex there is a delay of the order of half a minute before maximal potentiation is attained after a relatively long tetanus at high frequency (Lloyd, 1949). On the other hand, this delay is very much shorter in the two spino-cerebellar tracts (Oscarsson, 1957*b*, Fig. 11). Presumably this indicates that there is much less development of synaptic depression, which may be related to a more efficient mobilization of transmitter in the presynaptic terminals.

TABLE 3. Two patterns ((1) and (2)) of convergence of monosynaptic Group I excitation and disynaptic Group I inhibition in VSCT neurones. The columns indicate the effects from flexors (F) and extensors (E) working at various joints. The nerves used for testing and representing these functional groups of muscles are also indicated (abbreviations on p. 487). Toe flexors and extensors refer to the muscle groups with flexor and extensor function (physiological flexors and extensors). Monosynaptic excitation is indicated by +, disynaptic inhibition by −, and the almost complete lack of either effect by 0. (+) indicates that the excitation occurred only in about half the number of cases. The effects from sartorius (SA) were not assessed for the neurones conforming to pattern (2). This table is based both on the present investigation and on the results obtained previously (Oscarsson, 1957*b*), and shows the convergence found in the majority of more than 100 investigated VSCT units

	Hip F SA	Hip E ABSM	Knee F PBST, GR	Knee E Q	Ankle F PTF	Ankle E GS	Toe F PTF	Toe E FDHL, PL
(1)	0	+	+	−	0	+	0	+
(2)	?	(+)	0	+	0	+	0	+

The convergence of monosynaptic excitation to motoneurones and DSCT neurones is usually restricted to a few nerves innervating synergist muscles. On the contrary, convergence of excitation from various nerves to VSCT neurones was, as a rule, very extensive. By combining in Table 3 the results from a previous (Oscarsson, 1957*b*) and from the present investigation, some general principles of the organization of convergence have appeared, which are based on recording from more than 100 VSCT neurones. Most of the VSCT neurones belonged to one of two groups: the neurones of one group, (1) of Table 3, were activated from hip extensors, knee flexors, and ankle and toe extensors; the other group (2) consists of neurones activated from knee, ankle, and toe extensors, and sometimes also from hip extensors. A distinct feature is the lack of co-activation from antagonist muscles. In this respect the organization of convergence to

VSCT neurones is similar to that found with both motoneurones and DSCT neurones (Holmqvist *et al.* 1956; Eccles *et al.* 1957*b*; Eccles & Lundberg, 1958*b*). The remarkable degree of excitatory convergence is well illustrated in Table 3. Neurones conforming to either of the two groups usually received excitation from one synergic muscle group at each of the hip, knee, ankle, and toe joints. Presumably each group of VSCT neurones is mainly activated from the muscles that contract together in the execution of a certain movement or the maintenance of a certain posture. Strong adequate activation of tendon organs in a single muscle is often insufficient to discharge VSCT neurones (Oscarsson, 1960). This would indicate that the VSCT neurones carry information, not concerning changes of tension in single muscles or muscle groups acting at single joints, but concerning stages of movement or position of the whole limb, as suggested by Oscarsson (1960). Table 3 shows also the occurrence of disynaptic inhibition. It was in general uncommon, though it was almost always evoked by the quadriceps nerve in VSCT cells of group (1). Since these cells are excited from the antagonist (PBST) muscles, there would appear to be a reciprocal organization. However, disynaptic inhibition did not occur from any of the other antagonist muscles (hip, ankle, and toe flexors). Furthermore, the inhibition from the quadriceps nerve was not limited to units activated from the PBST; it occurred in five VSCT cells of group (2) that were activated monosynaptically from the quadriceps nerve itself. It can be concluded that the functional significance of this I a disynaptic inhibition is obscure.

Usually the inhibition from I b afferents was tri- or polysynaptic. There was no indication that it was organized according to a reciprocal pattern. Possibly this polysynaptic inhibition is subsidiary to the inhibition from Group II and III muscle afferents and skin afferents. It has been suggested that inhibition from the latter afferents mediates information of flexor reflex patterns (Holmqvist *et al.* 1960*a*), and it is known that the I b afferents have a segmental action on motoneurones conforming to these patterns (Eccles *et al.* 1957*c*). The possible significance of the convergence of monosynaptic excitation, probably conveying information of movement or posture, and the polysynaptic inhibition, probably representing flexor reflex patterns, has been discussed recently (Oscarsson, 1960).

SUMMARY

1. Cells in the L4–L5 segments of the cat cord were identified as VSCT cells if they were excited antidromically by stimulation of the dissected contralateral spinal half, and monosynaptically by stimulation of ipsilateral Group I muscle afferents.

2. The spike potential of VSCT cells resembles that of motoneurones in being composed of IS and SD components, but their coupling is closer and the following hyperpolarization shorter, than in motoneurones.

3. Excitatory post-synaptic potentials (EPSPs) were recorded when spikes were suppressed by depolarization arising through deterioration of the VSCT cells. Brief monosynaptic EPSPs were evoked by Group I stimulation in 37 VSCT cells. In 34 cells Ib volleys, in 1 cell Ia volley and in 2 cells both Ia and Ib volleys were responsible for the EPSPs.

4. Ib EPSPs increased in size during repetitive stimulation at frequencies above 1–10/sec. There was no significant decrease in size at such frequencies of stimulation. The potentiation was as much as 50–100% at 50–80/sec. Ia EPSPs decreased in size during repetitive stimulation just as Ia EPSPs in motoneurones.

5. VSCT cells were inhibited both by Ia and Ib muscle afferents. Latencies of IPSPs show the former to be disynaptic and the latter polysynaptic.

6. Patterns of convergence of monosynaptic Group I excitation and disynaptic Group I inhibition are described and some common patterns are presented in tabular form. The functional significance of these patterns is discussed.

7. Volleys in skin afferents and Group II and III muscle afferents of all tested nerves produced IPSPs in VSCT cells.

8. VSCT cells are excited by descending fibres in the ipsilateral ventral quadrant of the spinal cord and inhibited by descending fibres in the ipsilateral dorsolateral funiculus. The excitation was monosynaptic, but inhibition was mainly disynaptic although monosynaptic and polysynaptic inhibition occurred. The inhibition markedly increased during repetitive stimulation.

9. There is a discussion of the factors responsible for the very high frequency at which impulses can be transmitted along the VSCT pathway.

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SYNAPTIC ACTION OF GROUP I AND II AFFERENT FIBRES OF MUSCLE ON THE CELLS OF THE DORSAL SPINOCEREBELLAR TRACT

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The synaptic connexions made by the primary afferent fibres of muscle give special opportunity for investigation of fundamental problems in neurophysiology. Such problems relate both to the detailed mechanisms of operation of individual synapses, and, more generally, to the organization of the functional connexions made by the afferent fibres from various types of receptors in the muscle. In the former category are considerations both of the time course of transmitter action (see Curtis & Eccles, 1959) and of the supply of transmitter during sustained stimulation at various frequencies (see Curtis & Eccles, 1960). In the latter category are several concepts relating to the manner in which the specific patterns of synaptic connexions are originally developed, and the role of the postulated chemical specificities in this development (Eccles & Oscarsson, 1961). A further general problem relates to the manner in which the information signalled by receptor organs from muscles of diverse function is transmitted and integrated.

Though many valuable data have been derived from studies on the discharges of nerve cells under controlled conditions of synaptic stimulation, intracellular recording of the membrane potential changes produced by synaptic action (the excitatory and inhibitory postsynaptic potentials, EPSP and IPSP) give much more precise and detailed information relating to all the problems outlined above. Already this claim has been amply demonstrated with intracellular investigations on motoneurones. Hitherto there has been one preliminary intracellular study on the cells of the dorsal spinocerebellar tract (DSCT cells) (Curtis, Eccles & Lundberg, 1958); but the present investigation is also closely related to the preceding systematic studies on the impulses discharged from the DSCT cells

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(Laporte & Lundberg, 1956; Laporte, Lundberg & Oscarsson, 1956*a*, *b*; Lundberg & Oscarsson, 1956, 1960; Holmqvist, Lundberg & Oscarsson, 1956; Lundberg & Winsbury, 1960).

METHODS

The experiments were performed on cats under light pentobarbitone anaesthesia. The dissection and experimental arrangements were similar to those of the preceding paper (Eccles, Hubbard & Oscarsson, 1961) with the exception that the micro-electrode was inserted at the L3 segmental level of the spinal cord. As previously reported (Curtis *et al.* 1958), the DSCT cells were found at a depth of about 2 mm from the dorsum of the cord and close to the mid line. They were identified by two criteria in addition to location, antidromic invasion by a descending volley on the ipsilateral side of the cord and monosynaptic excitatory action by Group I afferent volleys from muscle. The muscle afferent nerves and their abbreviations were as follows: the knee extensor, quadriceps, Q; the knee flexors, posterior biceps + semitendinosus, PBST; the hip extensors, anterior biceps + semimembranosus, ABSM; the ankle extensors, gastrocnemius + soleus, GS; the plantar extensors of digits, plantaris, PL; and flexor digitorum longus and flexor hallucis longus, FDHL; the dorsi-flexors of ankle and digits, collectively known as the pretibial flexors, PTF.

RESULTS

Synaptic excitatory action

By choosing appropriate strengths of stimulation it is often possible to excite almost all the Ia afferent fibres of some muscle nerves and yet excite few if any Ib fibres (Bradley & Eccles, 1953; Eccles, Eccles & Lundberg, 1957*a*; Laporte & Bessou, 1957). By recording the afferent volley from the dorsal root and employing the double-volley technique it is possible to measure the approximate sizes of the Ia and Ib afferent volleys for each strength of stimulation, and hence to plot the Ia and Ib composition against stimulus strength, as in Fig. 1J. Figure 1B–H are samples of intracellular potentials of a DSCT cell that were elicited a few minutes earlier in response to graded stimulation of the PBST nerve. The respective strengths of stimulation are marked by the appropriately labelled arrows in Fig. 1I, and the inset records of Fig. 1B–F show at a faster sweep speed the potential produced by each afferent volley in the dorsal root.

A very weak stimulus, that can be presumed to excite only Ia afferent fibres, produced an EPSP adequate to evoke a spike in the partly depolarized cell (Fig. 1B). There was a slight underswing after the spike, which was even larger after the antidromically evoked spike in A, and which is characteristic of the action potentials of depolarized neurones (Coombs, Eccles & Fatt, 1955*a*). With progressive increase in the stimulus strength (C–H), it is seen that this underswing was replaced by a residual depolarization that had an incrementing phase until as long as 5 msec after the initial onset of the EPSP. In measuring the size of this residual depolarization the underswing following the antidromic spike (A) was taken as the

base line. When the mean size of this residual depolarization is plotted against the stimulus strength in Fig. 1I, it is seen that much of it was produced by impulses in fibres that are identifiable as Ib fibres. When the Ib contribution was well developed, the residual depolarization was often

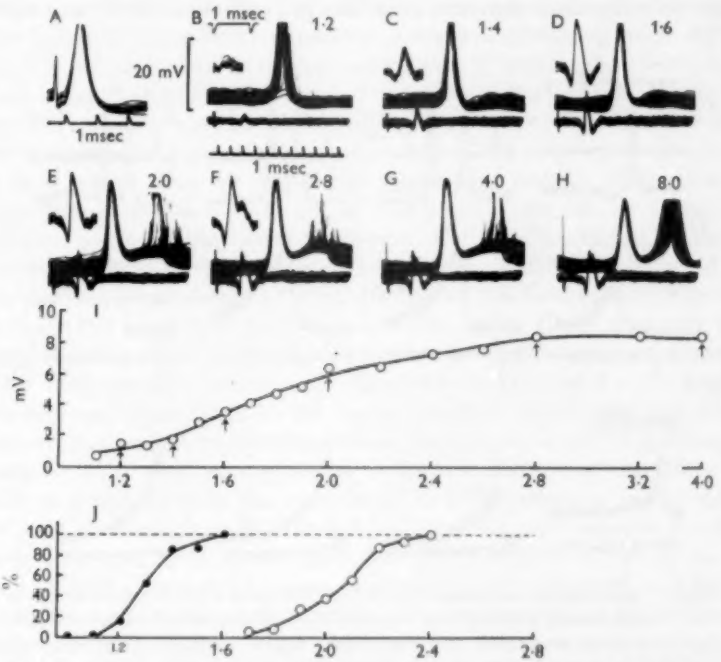

Fig. 1. Intracellular recording of spikes and EPSPs of a DSCT cell with a membrane potential of −30 mV. All records are formed by the superposition of about 50 faint traces, the repetition rate being about 50/sec. A shows the antidromic spike potential evoked by a descending impulse in the ipsilateral half of the cord. In B–H the stimulus intensity to the posterior biceps-semitendinosus nerve was increased progressively, the strength relative to threshold being indicated on each record. The potential produced by each afferent volley at the entry of L7 dorsal root is shown below the intracellular traces on the same time scale, and it is also shown at much faster sweep in the inset records of B–F (note time scale inset in B). Same voltage scale for all intracellular records. Note faster time for A. I: potentials of the intracellular records 3 msec after the onset of the spike plotted against the stimulus strengths, relative to threshold, as abscissae. The base line from which these residual depolarizations are measured is the potential recorded also at 3 msec after the onset of the antidromic spike (A). The arrows indicate points derived from the records B–G. J: also plotted on the same abscissae are the percentages of Ia and Ib afferent impulses excited at the various strengths and measured by the double-volley technique (see Eccles *et al.* 1957*a*).

large enough to cause a second impulse discharge (E–F). When the stimulus was strong enough to excite most of the Group II fibres (Fig. 1H), it invariably evoked the second impulse.

In Fig. 1 the residual depolarization is already in evidence during the declining phase of the spike, so its latency cannot be measured. Hence it could be maintained that this large late production of EPSP was due to

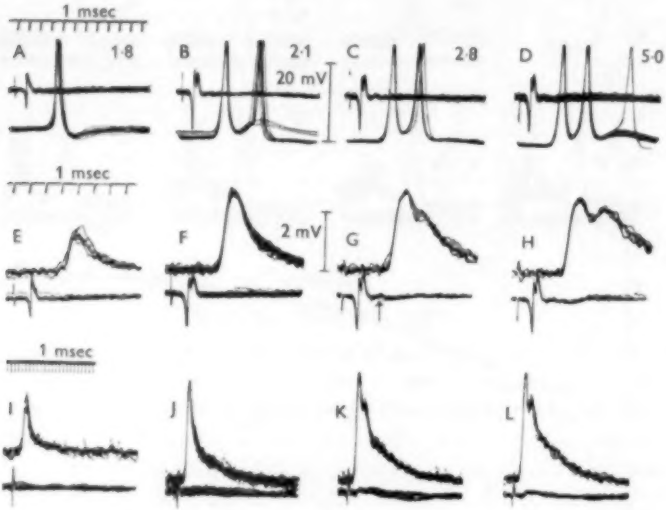

Fig. 2. Intracellular recording of spikes and EPSPs of a DSCT cell as in Fig. 1. In A–D the resting potential was −54 mV. Later it was lower (−40 to −30 mV) and the spike mechanism was suppressed. Similar strengths of stimuli were applied to the posterior biceps-semitendinosus nerve for the records of each column, e.g. for A, E, I; B, F, J, etc. I–L are similar to E–H and at same amplification, but photographed at a much slower sweep speed, as indicated by time scales. Note the records of the afferent volley entering the cord at L7 level. An arrow in G marks the summit of the small spike potential of the Group II afferent impulses. All records were formed by the superposition of about ten traces and the repetition rate was about 10/sec. Note different sweep speeds and voltage scales for A–D and for E–H.

delayed synaptic bombardment, the Ia and Ib volleys being relayed through one or more interneurones. The alternative explanation would be that the synaptic bombardment had no more temporal dispersion than would be expected for monosynaptic activation, and that after the spike potential the synaptic depolarization was rebuilt by the residual transmitter, just as occurs with most synapses (Curtis & Eccles, 1959; Hagiwara, Watanabe & Saito, 1959; Nishi & Koketsu, 1960; Fadiga & Brookhart, 1960).

Usually, when the recording electrode remained intracellularly for a few minutes the DSCT cell became so depolarized that the spike mechanism was completely inactivated, and it was then possible to reinvestigate the conditions giving residual depolarization, without complication by the spike potential, as in Fig. 1. For example, in Fig. 2 B the residual depolarization resembled that in Fig. 1 E in often causing a second spike discharge. After failure of the spike a comparable strength of stimulus gave an EPSP (Fig. 2 F), which showed no sign of a later phase of synaptic bombardment; it was larger than in Fig. 2 E but had an identical time course. However, when the stimulus was increased into the Group II range (G, H), there was a second wave on the declining phase of the EPSP with a latency about 1·5 msec longer than for the initial wave. This latency differential is adequately explained by the longer conduction time for the Group II impulses, which, in the dorsal root record of G, can be seen to be about 1·0 msec longer than the conduction time for I b. Possibly there would be a further additional delay in the conduction up the dorsal columns to the L 3 segmental level, so at least the main part of the EPSP added by the Group II volley would be monosynaptically evoked. The later components added with stronger stimulation (H) would be delayed by the longer impulse conduction times in the higher-threshold fibres, but the delay could in part be due to synaptic relays through polysynaptic pathways. It will be noted that, corresponding to the late addition of the Group II EPSP in G and H, there was increase in the EPSP after the second spike in C, and particularly in D, where it was large enough to evoke the discharge of a third spike in one trace. The slow records I–L correspond to those of E–H. Even the smallest response, I, was composed of a rapid initial phase, about 4 msec in duration, and a small residuum that decayed slowly over many milliseconds; and there was a total duration of about 30 msec for the EPSP in the larger response, J, which had no appreciable Group II contamination (cf. F). As would be expected, the EPSP exhibited a still slower decay in K and L, where the later Group II components were added.

The two-phase decay was a regular feature of the EPSPs of depolarized DSCT cells. A particularly good example is illustrated in Fig. 3, because this cell was monosynaptically activated by Group I b fibres from gastrocnemius-soleus, with no trace of either I a or II activation. In A the large Group I a volley was associated with a just-threshold EPSP, which would correlate with the stimulus being just at I b threshold. The large EPSP of C was evoked by a maximum I b volley, while in D the addition of Group II did not cause the development of a late EPSP wave, as in Fig. 2 G, H; the decline relative to C was attributable to a deterioration of the recording conditions. The two-phase decay of the EPSP is well illustrated in the

slower records of E–H, where E corresponds to B and G to C. The remarkably slow decline of the later stages of the EPSP is shown in I, where it could be detected for as long as 40 msec, and even longer durations occurred after repetitive stimulation in J to P.

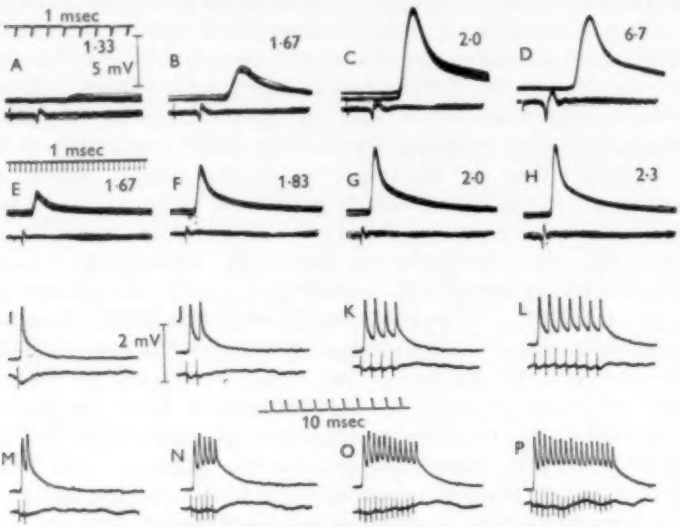

Fig. 3. Intracellular recording of EPSPs from a DSCT cell as in Fig. 2, E–L. Afferent-volley records are below all intracellular records. In A–H a stimulus was applied to the gastrocnemius-soleus nerve at the indicated strengths relative to threshold, A–D being photographed several times faster than E–H. All records are formed by the superposition of about ten faint traces. In I–P the sweep speed was still slower, with brief repetitive stimulation at 140/sec in J–L and at 280/sec in M–P. The stimulus strength to the gastrocnemius-soleus nerve was maximal for Group I. Separate voltage scales for A–H and I–P.

Since primary afferent fibres make very large synaptic endings on DSCT cells (Szentagothai & Albert, 1955), it would be expected that large unitary synaptic potentials would be a prominent feature. Two techniques were employed in attempting to discover the size of the 'grain' of synaptic activation. Under favourable conditions, with an afferent volley evoking a large and stable EPSP (Figs. 2 and 3), gradual increase of the volley revealed that there was a large number of steps in the size of the EPSP. Up to twenty steps could be recognized with some DSCT cells, a sample of such steps being illustrated by the graded series in Fig. 4F–O. The expected large steps of more than 1 mV were rarely encountered. The alternative procedure was simply to photograph a large number of traces

of the spontaneous synaptic noise that is generally present with intracellular recording (cf. Brock, Coombs & Eccles, 1952). The unitary EPSPs so observed (Fig. 4A–E) have often been larger than with motoneurones, ranging up to 3 mV. However, the disadvantage of this method is that in the conditions of our experiments most of the primary afferent fibres will be severed from their receptors in the muscle and the synaptic noise is likely to be largely due to the synaptic endings of interneurones, i.e. the method samples the unitary EPSPs produced by the small synapses rather than those produced by the giant synaptic endings of the primary afferent fibres (Szentagothai & Albert, 1955). Evidently no correlation is yet possible between the histological and the physiological investigations.

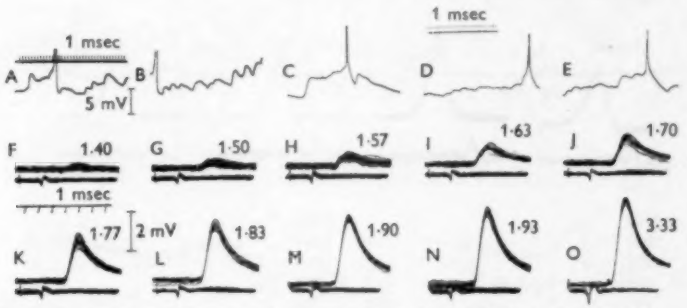

Fig. 4. Sizes of unitary synaptic potentials of DSCT cells. A, B and C–E are samples of synaptic noise and occasional spike discharges spontaneously produced in two DSCT cells. With both, A, B and C–E the resting potential was about −30 mV. F–O are intracellularly recorded EPSPs from another cell produced by progressively increasing stimuli to the gastrocnemius-soleus nerve, as indicated by the strengths relative to threshold. Each record was formed by superposition of about ten faint traces. Note the records of afferent volleys below the EPSPs; also separate time scales for the records from each of the three DSCT cells, and separate potential scales for A–E and for F–O.

Repetitive synaptic activation

DSCT cells differ from motoneurones in being able to discharge impulses at very high frequencies. For example, with recording from single DSCT fibres Holmqvist *et al.* (1956) found that the discharge of impulses followed frequencies of afferent stimulation even as high as 500/sec, which is at least ten times as high as the performance of motoneurones. VSCT cells are also remarkable for the high frequency at which they can discharge in response to synchronized synaptic stimulation (Oscarsson, 1957). At least in part this high frequency of response is attributable to the effect of repetitive stimulation in potentiating the sizes of the individual EPSPs produced by the Ib volleys (Eccles, Hubbard *et al.* 1961). In two VSCT

cells aberrant monosynaptic connexions from Ia afferent fibres displayed a frequency–response curve that resembled the Ia curves with motoneurones (Curtis & Eccles, 1960), which is in contrast to the large potentiations exhibited by Ib synapses on the same cells (Eccles, Hubbard *et al.* 1961). An investigation of the EPSPs with repetitively activated DSCT cells allows a further comparison between the performance of Ia and Ib afferent impulses on the same type of cell.

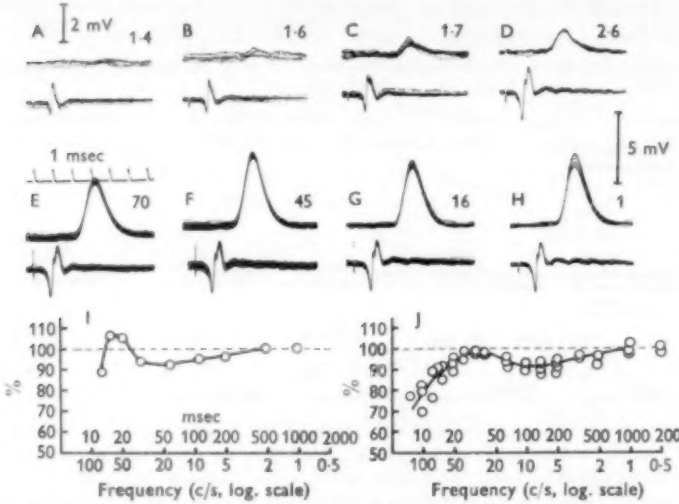

Fig. 5. The effect of frequency on size of EPSPs generated by Ib afferent volleys. In A–H are EPSPs produced by hamstring afferent volleys and recorded intracellularly from a DSCT cell with a membrane potential of −24 mV. Potentials produced by the afferent volley as it enters the cord at L 7 dorsal root are below the intracellular records. The EPSP is clearly related to the Ib component of the afferent spike in the series of A–D with graded stimulus strengths, as indicated relative to threshold. In E–H are EPSPs evoked by maximum Group I volleys at the frequencies of repetition marked on each record (in c/s) and recorded after a steady state had been reached. Same amplification for intracellular records, A–D, and E–H respectively. Note greatly improved recording for E–H. I: ordinates, sizes of the EPSPs calculated relative to the size of the slowest frequency; abscissae, frequency or volley intervals (log. scale). J: a similar series for the EPSPs evoked in another DSCT cell by Group Ib gastrocnemius-soleus volleys.

Figure 5A–D shows that a hamstring (PBST + ABSM) afferent volley evoked an EPSP entirely by its Ib impulses. During repetitive stimulation the sizes of the EPSPs produced by the successive impulses rapidly attained a steady state, just as occurs with motoneurones (Curtis & Eccles, 1960). The size of this steady state EPSP at various frequencies is illustrated in

Fig. 5E–H and plotted in I. When the frequency of activation was increased from 1/sec up to 16/sec, the EPSP was depressed (G, H and plotted points of I), but at frequencies around 50/sec the EPSP was larger even than at 1/sec (F and the plotted points of I), while at still higher frequencies (E, I) there was depression. The depression at frequencies around 5–10/sec was always observed with the Ib EPSP of DSCT cells, and the potentiation at higher frequencies sometimes failed to lift the EPSP up to its size at the slowest frequencies (Figs. 5J, 7A).

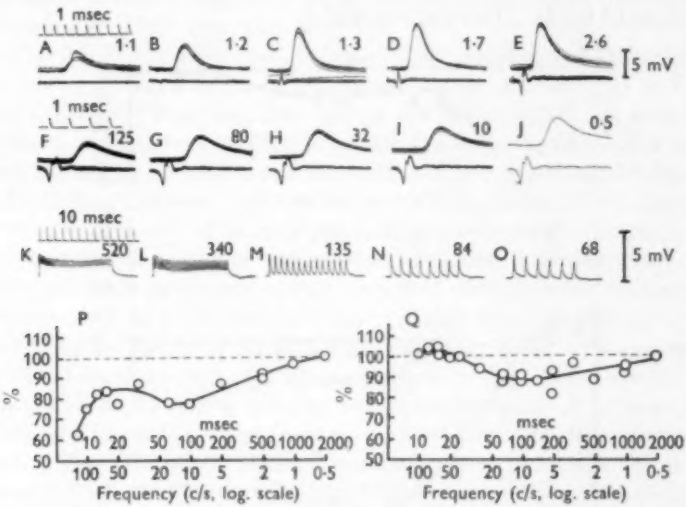

Fig. 6. The effect of frequency on size of EPSPs generated by Ia afferent volleys. Records A–J resemble Fig. 5A–H, being EPSPs evoked by hamstring volleys in the same experiment from a different DSCT cell with membrane potential, −20 mV. The Ia is effective and not the Ib component, as is shown by the graded-strength series A–E. In A–J the afferent volley records from the L7 dorsal root are shown just below the corresponding intracellular records. F–J are specimen records at the indicated frequencies (c/s) and after the steady state had been reached for the points of P, which are plotted as in Fig. 5I. K–O show repetitive responses at the various frequencies as indicated (in c/s). Note that summation of residual depolarization is much less prominent than for the Ib EPSPs in Fig. 3J–P; also much slower sweep speed for K–O. Q: another frequency series for EPSPs evoked in a different experiment by Ia plantaris volleys.

A complementary investigation on the EPSP produced by the Ia afferent fibres of the same nerve (PBST + ABSM) in the same experiment is illustrated in Fig. 6. The series A–E shows that the EPSP was produced in response to stimuli just above threshold (A, B), and that a maximum (C) was attained by a stimulus that appeared to be below the Ib threshold.

Repetitive stimulation at the various frequencies illustrated in F–J and plotted in P shows that the relationship between frequency of stimulation and size of EPSP corresponds closely to that observed with the monosynaptic EPSPs of motoneurones (Curtis & Eccles, 1960). There was a minimum at about 10/sec and a slight increase at higher frequencies, up to about 50/sec, but a decline at still higher frequencies. Sometimes, as with motoneurones, the potentiation at frequencies of about 50/sec lifted the EPSP above its size at the slowest frequencies (Fig. 6Q). The attainment of a steady state during repetitive stimulation at a wide range of frequencies is illustrated for Ia EPSPs in Fig. 6K–O.

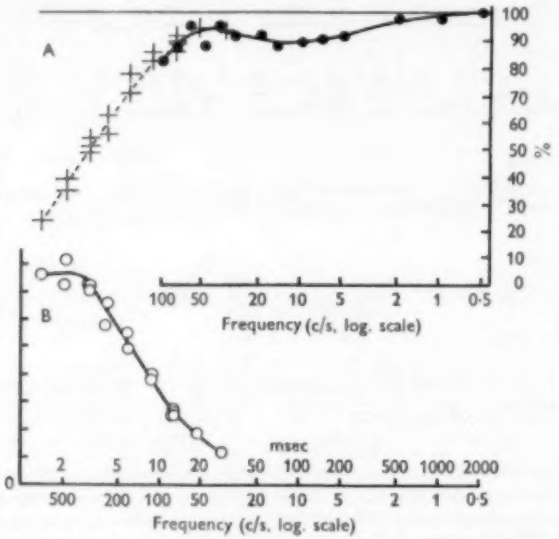

Fig. 7. Effect of frequency on efficiency of synapses. EPSPs are produced by maximum Ib gastrocnemius-soleus volleys and intracellularly recorded from a DSCT cell (specimen records being seen in Fig. 3). In A the size as percentage of the slowest frequency response (scale to right) is plotted against the frequency, as in Fig. 5I. The crosses are points from a later series than the dots, and form the values for the calculations giving the points plotted in B. In B the sizes of the EPSPs are calculated at the steady-state condition for frequencies from 30 to 600/sec, and the product of size and frequency is plotted as ordinate (arbitrary scale on left) on the same frequency and volley-interval scale as in A. For details see text.

The frequency–response curves for Ib activation of DSCT cells in Figs. 5I, J and 7A differ from the curves for VSCT cells (Eccles, Hubbard *et al.* 1961) in that the depression at moderate frequencies was small or absent with the latter cells. This depression is characteristic of all DSCT cells so

investigated, as also is the potentiation at higher frequencies (about 50/sec). Thus in their frequency response characteristics the I b EPSPs on DSCT cells resemble the I a EPSPs on motoneurones (Curtis & Eccles, 1960) and DSCT cells (Figs. 6 P, Q) rather than the I b EPSPs on VSCT cells. However, there has been a general tendency for the I b EPSPs of DSCT cells to show a larger potentiation at high frequencies (about 50/sec) than the I a EPSPs on these cells.

There has been no systematic study of Group II action at different frequencies. However, Fig. 8 I–L shows that the Group II EPSP was more potentiated at 70/sec than the I a EPSP.

In Fig. 3 J–P it is seen that the sizes of the successive EPSPs decline considerably during a rapid repetitive stimulation to reach, as in P, for example, a steady state less than half of the initial EPSP. At such high frequencies the successive EPSPs are always superimposed on a background formed by summation of the residua of the preceding EPSPs, and each EPSP has to be measured relative to this background. If it be assumed that the size of the EPSP is approximately proportional to the amount of transmitter that is acting on the neurone (cf. Curtis & Eccles, 1960), it is possible to obtain an approximate measure of the amount of transmitter that is liberated by repetitive stimulation in unit time by multiplying the frequency by the size of the EPSP during the steady state. As so measured, the rate of liberation of the transmitter is seen in Fig. 7 B to increase to a maximum as the frequency is raised to 300/sec, levelling off between 300/sec and the highest testing frequency at 660/sec. At this maximum plateau in Fig. 7 B the rate of liberation is more than twice as great as at a frequency of 100/sec. The curve of Fig. 7 B is typical of the curves obtained for several DSCT cells and closely resembles the curves given by motoneurones (Curtis & Eccles, 1960).

Convergence

In 58 DSCT cells there has been an extensive investigation of the monosynaptic excitation by Group I volleys from many muscles, and an attempt to discriminate between I a and I b components both by threshold and conduction-velocity criteria (cf. Bradley & Eccles, 1953; Eccles *et al.* 1957*a*; Laporte & Bessou, 1957). This investigation (see Table 1) is a further development of several previous studies in which the criterion of activation was the discharge of impulses from the DSCT cells, recorded in the DSCT tract either as a mass discharge or intracellularly from individual nerve fibres (Laporte *et al.* 1956*a*, *b*; Laporte & Lundberg, 1956; Lundberg & Oscarsson, 1956, 1960; Holmqvist *et al.* 1956; Lundberg & Winsbury, 1960). There were also a few DSCT cells in which convergence was studied by intracellular recording (Curtis *et al.* 1958).

With 22 DSCT cells of the present series there was monosynaptic excitation by Group Ia and not by Group Ib afferent impulses. In 17 this excitation was from one afferent nerve only (e.g. in the cell of Fig. 6 no Group I volley other than hamstring produced any EPSP) and in 5 two or three nerves participated, these sometimes being synergists, as in Fig. 8, but on two occasions a DSCT cell was excited by Ia volleys from antagonistic muscles—PBST and quadriceps in one, pretibial flexors, PTF, and PL+FDHL, in the other.

TABLE 1. Convergence of monosynaptic excitatory actions by Group Ia and Ib impulses on 58 DSCT cells, as enumerated in the columns. For details see text

	Ia only	Ib only	Ia+Ib
Totals	22	15	21
From one afferent nerve only	17	11	9
From more than one afferent nerve	5	4	12

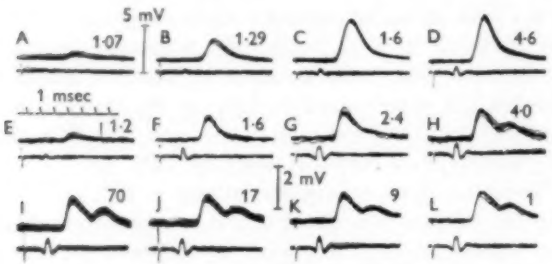

Fig. 8. Convergence of Group Ia and II impulses on a DSCT cell. A–D and E–H are EPSPs evoked in the same DSCT cell in response to graded stimulation of the PL and GS nerves respectively, the strengths relative to threshold being marked on each record. Note that, as partly illustrated in A–H, the EPSPs were evoked with stimuli just at Ia threshold and increased to a maximum with maximum Group Ia volleys. The afferent volley records from L7 dorsal root are below the corresponding intracellular records. The Group II EPSP is evident in H, but barely perceptible in D. The EPSPs evoked by maximum Group I and II volleys in the GS nerve at the indicated frequencies (c/s), are seen in I–L, the Group II EPSP being even potentiated at the highest frequency. Note potential scales for A–D and E–L.

With 15 DSCT cells there was monosynaptic excitation by Group Ib and not by Group Ia afferents. In 11 this excitation was from one afferent nerve only (e.g. in the cell of Fig. 2 no Group I volley other than PBST produced any EPSP) and in 4 from two or three nerves (for example, ABSM in addition to the GS responses in Fig. 3). There was one example of Ib volleys from antagonistic muscles monosynaptically exciting the same cell (GS, FDHL, PTF). Ib volleys from extensor muscles were far more effective than those from flexors, which corresponds with the

observations on the disynaptic excitation or inhibition of motoneurones by Ib impulses in the lumbosacral region (Eccles, Eccles & Lundberg, 1957*c*).

With 21 DSCT cells there was evidence of monosynaptic innervation from both Ia and Ib primary afferent fibres. Possibly some of these instances are attributable to unrecognized contamination of the presumed Ia volley by Ib impulses, and vice versa (cf. Eccles *et al.* 1957*a*; Laporte & Bessou, 1957); but this explanation seems most improbable with many examples, where the Ia and Ib responses were very well discriminated.

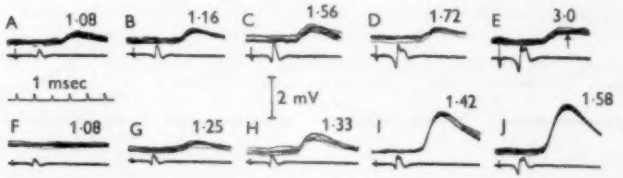

Fig. 9. Convergence of Ia, Ib and II afferent impulses on a DSCT cell. Increasing the stimulus to PBST nerve above Ia maximum in B up to Ib maximum in D produces no change in the EPSP, but a later addition due to Group II is seen in E at the arrow. In contrast an almost maximum Ia volley in ABSM nerve produces no EPSP(F) and in G–J the EPSP grows as the Ib volley increases to maximum in J. Note records of afferent volleys recorded in L7 dorsal root. Same time and amplification scales for all records.

With 9 cells there was merely Ia and Ib innervation from the afferent fibres of the same muscle nerve, as is illustrated in Fig. 1. With 12 cells the situation was more complex: in 4, Ia and Ib innervation from PBST and ABSM in various combinations (see Fig. 9); in 3, Ia excitation from synergic extensors and Ib from extensors of same or other joints; in 2, Ia excitation from extensors and Ib from flexors (see Fig. 10); and finally in 3, more complex arrangements that may perhaps be regarded as aberrancies.

When recording intracellularly from DSCT fibres Laporte *et al.* (1956*b*), Laporte & Lundberg (1956) and Lundberg & Oscarsson (1956, 1960) frequently observed that the afferent volley set up by stimulation of a muscle nerve generated the discharge of two impulses in quick succession. Sometimes this double discharge occurred with a stimulus strength submaximal for Group I; correspondingly, in the present investigation three DSCT cells responded by double spikes to muscle afferent volleys that were presumed to be entirely Group Ia; and in Fig. 1E there were often double spikes in response to a volley that should be purely Group I. Usually, however, the second impulse in the DSCT fibres occurred when the stimulus was increased to excite a considerable fraction of Group II, and

it was so early after the first impulse that the Group II volley was probably acting monosynaptically. Lundberg & Oscarsson (1956) further reported that this monosynaptic response to a Group II volley was produced only by those DSCT neurones in which the initial discharge was evoked by a Ia volley. This monosynaptic convergence of Group Ia and II fibres on the same nerve cell is of general interest in the problem of specificity of neuronal connexions (Eccles & Oscarsson, 1961), particularly as there is no evidence for this convergence on motoneurones (Eccles *et al.* 1957*a*; Hunt & Perl, 1960). Since intracellular recording from DSCT cells can detect

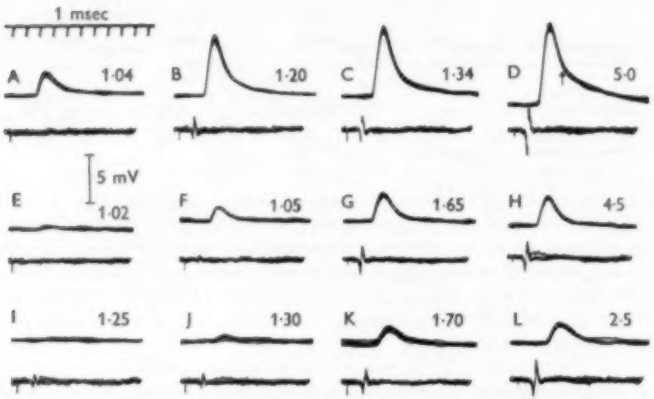

Fig. 10. Convergence of Ia and Ib impulses on a DSCT cell. EPSPs evoked in a DSCT cell by graded stimulation at indicated strengths relative to threshold of three nerves, GS in A–D, PL in E–H, Per in I–L. With the former two nerves EPSPs appear with just-threshold stimulation (A and E) and reach a maximum with submaximal Group I volleys; hence Group Ia activation is presumed. With the other nerve, I–L, an EPSP is not evoked until the Group I volley is quite large (J) and it increases up to maximum Group I in L, hence Group Ib activation is presumed. In D the arrow marks the onset of a small Group II EPSP that is superimposed on the declining phase of the Ia EPSP. Note records of afferent volleys recorded in L7 dorsal root. Same time and voltage scales for all records.

synaptic excitations that are far below the threshold for evoking discharges of impulses, it gives opportunity for rigorously testing these suggestions relating to specificity of synaptic connexions.

Altogether Group II volleys from muscle nerves were observed to evoke EPSPs in 33 DSCT cells; the latency was so brief that monosynaptic activation could be presumed in the great majority, though there is some uncertainty in the allowance for conduction time in the Group II primary afferent fibres. In 26 of these DSCT cells there was monosynaptic activation by a Ia afferent volley in the same nerve that gave the Group II

response (Figs. 1; 8E–H; 9A–E). Careful investigation by graded stimulation of the muscle nerves indicated that six of the remaining seven were activated only by Group Ib and II impulses, as is illustrated in Fig. 2, which is an association never observed by Lundberg & Oscarsson (1956). However, they reported Group II activation frequently (in 7 out of 18 in their Table 1) with DSCT cells excited by both Ia and Ib volleys; and this has been confirmed with the present intracellular investigation, where monosynaptic Ib activation occurred in 12 of the 27 DSCT cells excited by both Group Ia and II volleys (cf. Table 2). In 7 of these 12 cells the Ib

TABLE 2. Excitatory innervation of 33 DSCT cells by Group II afferent impulses in relation to Ia and Ib monosynaptic excitation, as enumerated in the columns. For details see text

	Ia only + II	Ib only + II	Ia + Ib + II
Totals	15	6	12
Both Group I and II in the same afferent nerve only	11	5	7
Group I and II in more than one afferent nerve	4	1	5

activation was produced from the muscle nerve that provided the Ia and II activation (Fig. 1), while in 4 of the remaining 5 cells the Ib activation was produced from a different muscle nerve (Fig. 9). There is another example in Fig. 10, where the GS Group II volley was responsible for the small wave that in D slowed the decline of the Ia EPSP from the arrow onwards, whereas J–L shows Ib activation from the pretibial flexors. In the exceptional cell Ib and II innervation from FDHL nerve was combined with Ia from GS nerve. There has been no instance of monosynaptic Group II activation from a muscle nerve that had no Ia or Ib excitatory action.

In summary, it can be stated that monosynaptic Group II activation of a DSCT cell was always associated with Group Ia and/or Group Ib monosynaptic excitation from that same muscle nerve. Sometimes there was also monosynaptic excitation of that same cell by Group Ia and/or Group Ib impulses from other muscle nerves. Group II connexions predominated on DSCT cells monosynaptically excited from Group Ia volleys, but many such cells (16 out of 43) had little or no Group II innervation (Fig. 6). An even larger proportion (9 out of 15) of DSCT cells monosynaptically activated by Group Ib volleys and not by Group Ia received no innervation from Group II (cf. Figs. 3; 4F–O; 5).

Inhibition

The technique of recording impulses in DSCT fibres allows inhibitory action on DSCT cells to be detected by the suppression or depression of their discharges (Laporte *et al.* 1956*b*; Lundberg & Oscarsson, 1956;

Holmqvist *et al.* 1956). In general it was found that inhibition was principally exerted from muscles antagonistic to the muscle providing the most effective Group I excitation, but the convergence of inhibitory action on DSCT cells was found to occur from the Group I afferent fibres of many other muscles (Holmqvist *et al.* 1956). It is now recognized that inhibition of motoneurones is effected by two entirely different mechanisms; one, called presynaptic inhibition (Frank & Fuortes, 1957), results from depression of presynaptic excitatory action by a special synaptic mechanism that depolarizes presynaptic fibres (Eccles, Eccles & Magni, 1960*a*; Eccles, 1961*a*; J. C. Eccles, F. Magni and W. D. Willis, unpublished observations); the other, post-synaptic inhibition, is due to inhibitory synaptic action that directly antagonizes the synaptic excitatory action on the post-synaptic membrane (Coombs, Eccles & Fatt, 1955*c*; Eccles, 1961*a*, *b*). The method of testing for inhibition by suppression of impulse discharge does not discriminate between these two possible modes of inhibitory action. The very extensive convergence of inhibitory action on a single DSCT unit (Holmqvist *et al.* 1956) could possibly be mediated by presynaptic inhibition, for this type of inhibition would be greatly potentiated by their technique of employing repetitive inhibitory volleys at 100/sec (Eccles *et al.* 1960*a*). Intracellular recording from DSCT cells should allow discrimination between these two modes of inhibitory action, for presynaptic inhibition has no direct action on the post-synaptic membrane (Frank & Fuortes, 1957; Frank, 1959), whereas post-synaptic inhibition is revealed by the post-synaptic hyperpolarization, the inhibitory post-synaptic potential (IPSP).

Post-synaptic inhibition

The investigation has been restricted to IPSPs produced by Group I volleys and having latencies which indicated a pathway that was not longer than trisynaptic. Curtis *et al.* (1958) found that the latency of the IPSP of a DSCT cell was about 0·8 msec longer than that of a monosynaptic EPSP with a comparable presynaptic conduction distance. This latency differential corresponded precisely to that occurring with motoneurones (Eccles, Fatt & Landgren, 1956); hence it was postulated that there was an interneurone interpolated on the fastest inhibitory pathway to DSCT cells, just as with motoneurones (Eide, Lundberg & Voorhoeve, 1960; Araki, Eccles & Ito, 1960). In the present investigation Group I volleys from muscle evoked IPSPs in 19 DSCT cells. In all these cells Group I afferent volleys from other muscle nerves produced monosynaptic EPSPs with latencies that were on the average 0·8 msec briefer, the range for the differences extending from 0·6 to 1·3 msec. Thus there is confirmation of the postulate of a disynaptic inhibitory pathway from primary afferent

fibres of muscle to DSCT cells. There is similar evidence for the inhibitory pathway to VSCT cells (Eccles, Hubbard *et al.* 1961).

When surveying the patterns of inhibitory action on DSCT cells, the most striking feature is the large proportion of cells in which no disynaptic IPSP could be produced. Doubtless small IPSPs were overlooked in those cells that were discharging continuously at a fairly high frequency. Furthermore, the use of chloride-filled electrodes in most experiments may have caused IPSPs to be overlooked if Cl^- diffusion had caused the equilibrium potential for the IPSP to be close to the resting potential of the cell (see Coombs, Eccles & Fatt, 1955*b*). Nevertheless, it seems that post-synaptic inhibitory action on DSCT cells is much less commonly observed than would be expected from investigations on the discharge of impulses; hence there is the implication that presynaptic inhibition may make a significant contribution to DSCT cell inhibition.

Some DSCT cells resembled motoneurones in the relationship between the muscles from which they draw monosynaptic excitation and disynaptic inhibition. Since Group I a fibres are the only primary afferent fibres giving monosynaptic innervation to motoneurones, this comparison has first been made with DSCT cells that likewise have a monosynaptic Group I a activation.

Four DSCT cells have exhibited the reciprocal I a excitation and inhibition from antagonistic muscle groups that was first discovered by Lloyd (1946*a*, *b*) for motoneurones. With two there was monosynaptic excitation from a PBST I a volley with central latencies of 1·6 and 1·7 msec, and inhibition from a quadriceps I a volley with a central latency of 2·5 msec. The other two were excited from the pretibial flexors and inhibited from the GS and FDHL.

Ten DSCT cells were excited monosynaptically by Group I a from extensor and/or flexor muscle volleys, and inhibited by Group I b volleys either from their synergic muscles or from other muscles that were usually extensors (Fig. 11). This pattern of inhibitory action resembles that described for motoneurones (Eccles *et al.* 1957*c*). However, two of these ten cells differed from motoneurones in that they received monosynaptic I b excitation in addition to the I a. Since disynaptic inhibition is produced only if there is adequate convergence of excitatory action on the interpolated interneurone (Eccles & Lundberg, 1958*b*), it is possible that in some of these ten DSCT cells there was a potential I a inhibitory pathway, as in the preceding group, but it was effective only after summation with I b impulses that converged on the same interneurones. Thus with four out of these ten cells the I b inhibitory action was in part from the muscle antagonistic to that giving the I a excitation, as is illustrated in Fig. 11C, in which a GS volley exerted powerful inhibitory action on a cell excited

by Ia afferent fibres of the pretibial flexors (A, B). Figure 11F–G illustrates a further complication of this subgroup, for a Ia quadriceps volley produced both a disynaptic and polysynaptic IPSP.

The remaining five DSCT cells that exhibited disynaptic inhibitory action were monosynaptically excited only by Ib afferent fibres. In two

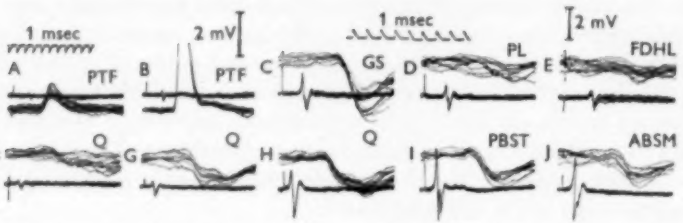

Fig. 11. EPSPs and IPSPs intracellularly recorded in a DSCT cell. A submaximal Ia PTF volley evokes an EPSP in A, and a larger Ia PTF volley an EPSP and truncated spike in B. C–E and H–J are IPSPs evoked by maximum Group I volleys in the nerves indicated on the records. F and G show that submaximal Group Ia volleys in quadriceps nerve evoke an IPSP with the initial disynaptic and the later polysynaptic component. Time scale for A, B shown above A; the other time scale is for records C–J. Note records of afferent volleys recorded in L7 dorsal root with negativity downwards.

of these cells the inhibition was largely by Ia afferent volleys: in one but not in the other it was from muscles antagonistic to the muscle having the monosynaptic action. The other three cells of this group received disynaptic inhibition largely by Ib impulses from a very wide field of muscles that were mostly extensors, which would correspond with the very effective Ib inhibitory action on motoneurones (Eccles *et al.* 1957*c*).

Presynaptic inhibition

Presynaptic inhibition of lumbosacral motoneurones as tested by depression of the monosynaptic EPSP is produced by Group I afferent volleys, chiefly Ib, and little if at all by Group II and III volleys (Frank & Fuortes, 1957; Frank, 1959; Eccles *et al.* 1960*a*). The largest effects are from knee and ankle flexor nerves, while extensor nerves are less active. The latency is several milliseconds, with a peak inhibition at about 10–20 msec, followed by a slow decline. Repetitive stimulation increases the effect for the first few impulses, so brief tetani provide the optimum conditions for display of presynaptic inhibition (Eccles *et al.* 1960*a*). These characteristics of presynaptic inhibition apply not only to EPSP depression in motoneurones, but also, as would be expected, to depression of the monosynaptic reflex (Eccles, Schmidt & Willis, unpublished observations).

A comparable test for the presynaptic inhibition of the DSCT pathway is provided by depression of the monosynaptic component of the mass discharge (Laporte *et al.* 1956*a*).

In one experiment, the mass discharge from a single gastrocnemius-soleus volley (Fig. 12A) was conditioned at various intervals by four stimuli at 300/sec to hamstring nerve at a strength submaximal for Group I as shown in the plotted curve (Fig. 12F). The mass discharge was

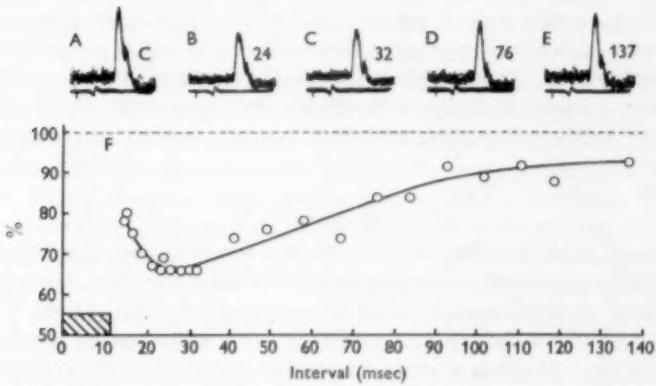

Fig. 12. Time course of presumed presynaptic inhibition. A–E are specimen records of DSCT mass discharge evoked by stimulation of GS and recorded from the dorso-lateral funiculus of the cord at L1–L2. A is the control, while B–E are records showing inhibition at various intervals (in msec) as indicated, following 4 conditioning volleys set up in the hamstring nerve by a stimulus strength submaximal for Group I. F is the curve of the time course of the inhibitory effect, the height of the mass discharge as percentage of control being plotted against time between the arrival of the first hamstring volley at L7 dorsal root and the beginning of the GS mass discharge. The duration of the brief conditioning tetanus is shown by crosshatched area.

depressed to about 65% of the control value when the interval between the dorsal root spike potential produced by the first conditioning stimulus to hamstring preceded the GS mass discharge by about 25 msec (Fig. 12B) and this depression declined over the next 100 msec or more. Other experiments gave similar results. They also showed that reducing the number of conditioning volleys from 4 to 2 or 1 diminished the inhibition. At an appropriate interval between conditioning and test volleys varying the strength of stimulation revealed that a maximum Ia volley had only a small inhibitory effect; a maximum Ib volley caused most of the inhibition, while a stimulus ten times threshold had no greater effect than did maximum Ib. When the effectiveness of various nerves in producing this

prolonged inhibition was compared, it was found that the largest inhibitions were produced by PBST and PTF volleys, while quadriceps and ABSM were less effective. In one experiment a brief GS tetanus reduced the mass discharge evoked by a hamstring volley to about 70% of the control, which is comparable to the maximum inhibition usually produced by PBST or PTF volleys; but otherwise the relative effectiveness of flexors and extensors in producing inhibition of DSCT cells was like that found for the monosynaptic activation of motoneurones.

It would appear, then, that the DSCT cells are inhibited in a pattern resembling closely that of presynaptic inhibition of lumbosacral motoneurones, which provides presumptive evidence that there is a presynaptic inhibitory action comparable with that on monosynaptic pathways. However, investigation by intracellular recording from DSCT cells is necessary before presynaptic inhibition can be regarded as established.

DISCUSSION

Curtis *et al.* (1958) observed that, when a Group I afferent volley monosynaptically generated an impulse in a DSCT cell, there was a considerable rebuilding of depolarization after the impulse (cf. Figs. 1B–F, 2A–D). It was postulated that this depolarization was produced by the delayed action of some of the excitatory transmitter that had survived for several milliseconds. An alternative postulate would be that there was delayed production of excitatory transmitter because of the late arrival of impulses that had traversed one or more synapses. This latter postulate was disproved in those experiments of the present series in which depolarization eventually caused failure of the spike. Afferent volleys that had been associated with a large post-spike depolarization (see Fig. 2B) were then seen to evoke EPSPs (Fig. 2F) characterized by the same early summit and regular decay that was exhibited by the EPSPs produced by afferent volleys just above threshold (E). EPSPs generated by delayed impulses would readily have been detected; as is illustrated in Fig. 2G, H, when the stimulus elicited the Group II volley in addition to the Group I volley.

It is not possible to calculate the time course of the transmitter action that generates the EPSPs of DSCT cells. With some nerve cells this has been done either by the difficult technique of voltage clamping (Terzuolo & Araki, 1961) or by calculation after measurement of the electric time constant of the membrane and allowance for any effects of electrotonic distortion (Curtis & Eccles, 1959; Nishi & Koketsu, 1960; Hagiwara *et al.* 1959). Recording conditions from DSCT cells have been too unstable to allow measurements of the electric time constant, though it may be assumed to be brief when deterioration leads to sufficient depolarization

to eliminate spike responses, as in Figs. 2, 3, and when the EPSPs display the initial very rapid decay. Hence it may be concluded that there is a very effective transmitter action after the spike in Figs. 1 and 2 and that the very prolonged tail of EPSP in Figs. 2 and 3 indicates that the residual transmitter action is prolonged at a considerable level for many milliseconds after the initial intense phase. Rall (1960) has suggested that, with monosynaptic activation of motoneurones, no transmitter survives for more than 2 msec, and that the rebuilding of EPSP after the spike is due to electrotonic spread from regions of the motoneurones not invaded by the spike. There are several grave objections to this interpretation (Eccles, 1961*c*), and in any case it could not account for the very large residual transmitter actions displayed by DSCT cells. Residual transmitter action is also indubitable with several other types of excitatory synapses (cf. Hagiwara *et al.* 1959; Fadiga & Brookhart, 1960; Eccles, 1961*b*), of which the EPSPs of Renshaw cells provide the most striking illustration (Eccles, Fatt & Koketsu, 1954; Eccles, Eccles, Iggo & Lundberg, 1961).

It may be asked if there is any structural feature of synapses on DSCT cells that could be correlated with the large residual transmitter action. Szentagothai & Albert (1955) showed that many of the synapses made by primary afferent fibres on DSCT cells are of 'giant' size, up to 10 μ in diameter; hence it would be expected that clearance of the transmitter out of the synaptic cleft by diffusion would be a relatively slow process taking many milliseconds (Eccles & Jaeger, 1958). Alternatively, elimination of the transmitter from the synaptic cleft might be delayed by diffusional barriers such as are assumed for Renshaw cells (Eccles, Eccles & Fatt, 1956; Curtis & Eccles, 1958). An additional feature of residual transmitter action that may be of significance in this context is that it was often much larger for EPSPs generated by I b afferent volleys than for the I a EPSPs.

As with motoneurones and VSCT cells (Curtis & Eccles, 1960; Eccles, Hubbard *et al.* 1961), the EPSPs of DSCT cells tended to reach a steady state when repetitively activated. The size of the I a EPSP of DSCT cells at the steady state exhibited a relation to frequency (Fig. 6P, Q) similar to that found with motoneurones and in the two investigations with VSCT cells that were activated by Group I a volleys. As the frequency was raised from a very low rate, the EPSP was depressed until a minimum of about 85% was reached with frequencies of about 10/sec. With further increase in frequency up to about 50/sec there was some increase in size, though usually there was failure to attain the level characteristic of the very slow frequencies. With the I b EPSP the frequency–size relationship differed only in that the minimum was less marked (average about 90% in Figs. 5I, J; 7A) and there tended to be a larger increase in the EPSP

as the frequency was raised from that giving the minimum to the maximum response at about 50/sec. In the small series of these frequency experiments on the Ib EPSPs of DSCT cells there was no example of the characteristic frequency–size relationship observed with VSCT cells; where with increasing frequency of activation there was initially little or no change in size, until at 1–10/sec there was potentiation which increased with frequencies up to about 50/sec, where the EPSP was even doubled in size (Eccles, Hubbard *et al.* 1961).

In comparison with Ia synapses the mobilization of transmitter is extremely efficient with the Ib synaptic endings on VSCT cells, which would suggest that this was a characteristic property of Ib synapses. The EPSPs evoked by repetitive Ib volleys in DSCT cells (Figs. 3J–P; 5 E–J; 7A) disprove such a generalization, though it seems that here also transmitter mobilization may be more efficient for the Ib than for the Ia synapses. Evidently the nature of the post-synaptic contact is also of importance in the establishment of high efficiency of transmitter mobilization in the presynaptic terminals. Thus Ib synapses on VSCT cells give very favourable conditions and on DSCT less favourable, while with Ia synapses on motoneurones, DSCT cells and VSCT cells conditions are usually still less favourable. However, there was a considerable variation in the performance of the Ia endings on motoneurones, there being three examples in which the EPSPs generated at about 50/sec were larger than at 0·3/sec (Curtis & Eccles, 1960). A transitional stage of considerable depression at moderate frequencies was observed with all of the Ia and Ib synapses, except with some Ib endings on VSCT cells where presumably the mobilization of transmitter was so efficient that at no frequency was depletion in evidence.

With motoneurones there are simple rules relating to the excitatory synaptic connexions made by primary afferent fibres from muscle, which were originally formulated by Lloyd (1943*a*, *b*; 1946*a*, *b*) and Laporte & Lloyd (1952): (i) monosynaptic connexions are established only by the Group Ia afferent fibres from muscle; (ii) these Group Ia monosynaptic connexions are made on a motoneurone only from the muscle innervated by that motoneurone and from synergic muscles; (iii) there is at least one interneurone on the excitatory synaptic connexions made by Ib afferent fibres, which are largely from antagonistic muscles. Intracellular recording techniques have so far failed to demonstrate any exception to rule (i), but there are many exceptions to rule (ii), particularly with motoneurones innervating the hip and knee joint (Eccles & Lundberg, 1958*a*), and the monosynaptic Ia activation of soleus motoneurones from vastocrureus is also a notable exception (Eccles, Eccles & Lundberg, 1957*b*). In addition, isolated examples of aberrances are occasionally observed with the very

discriminative investigation provided by intracellular recording; and nerve cross-union in young animals greatly accentuates these aberrant Ia monosynaptic connexions, which even seem to develop in an appropriate relationship to the newly acquired muscle function (Eccles, Eccles & Magni, 1960*b*). Finally, intracellular recording has failed to substantiate the second part of rule (iii) concerning Ib synaptic connexions, which in any case was based on contentious experimental evidence. The disynaptic or trisynaptic Ib excitatory connexions largely occur from extensor muscles to flexor motoneurones (Eccles *et al.* 1957*c*).

The excitatory synaptic connexions from muscle primary afferent fibres on DSCT cells do not conform to any one of these three original rules for motoneurones. Previous investigations established that Ia, Ib and II primary afferents all made monosynaptic connexions on DSCT cells (Laporte *et al.* 1956*a*, *b*; Lundberg & Oscarsson, 1956; Curtis *et al.* 1958; Lundberg & Winsbury, 1960). Besides providing ample confirmation of this basic finding, the present investigations have strongly supported the concept that any combination of Group Ia, Ib and II primary afferents can converge on a DSCT cell, i.e. that DSCT cells display no absolute selectivity in respect of afferent fibre type. Thus there have been many instances of DSCT cells monosynaptically activated from all three types of primary afferents, and also of the three other types of combination: Ia+II; Ia+Ib; Ib+II (Tables 1 and 2). The first combination (see Fig. 8) was also that commonly observed by Laporte *et al.* (1956*b*), and by Lundberg & Oscarsson (1956).

Lundberg & Winsbury (1960) employed a very discriminative method of adequate stimulation in order to test more rigorously the finding of Lundberg & Oscarsson (1956) that Group Ia and Ib fibres converge on the same DSCT cell. Their investigation was limited to DSCT cells that discharged impulses in response to Ia or Ib afferent volleys from soleus muscle. All the 29 DSCT cells investigated belonged to two categories, 15 discharging only in response to Ia volleys and 14 discharging only to Ib volleys. It was therefore suggested that there is no convergence of Ia and Ib impulses on DSCT cells, and that the convergence apparently demonstrated in the earlier experiments arose on account of contamination of the presumed Ia and Ib volleys with impulses of the other type (cf. Eccles *et al.* 1957*a*; Laporte & Bessou, 1957). In view of the present evidence for frequent convergence of Ia and Ib impulses on DSCT cells, it is important to realize that Lundberg & Winsbury tested only DSCT cells activated from soleus muscle and that their method would only detect Ia or Ib activation that was powerful enough to generate the discharge of an impulse. The present evidence relates to a wide variety of DSCT cells and is based on intracellular recording which detects even very

weak synaptic excitation. Furthermore, the possibility that Ia and Ib primary afferent fibres can form synapses on the same nerve cell would seem to be demonstrated beyond doubt with VSCT cells, where the presumed Ia and Ib pathways could be discriminated not only by threshold, but also by the very different frequency-response characteristics (Eccles, Hubbard *et al.* 1961). Nevertheless, it is admitted that some, but by no means all, of the presumed Ia–Ib convergence may be attributed to imperfections in the experimental procedures for discriminating Ia and Ib afferent volleys. It would be desirable to have confirmation by a combination of the techniques of adequate stimulation and intracellular recording. However, it can now be regarded as established that Ia and Ib primary afferent fibres can occasionally make synaptic endings on the same DSCT or VSCT cell. Though this combination of endings may be regarded as an aberrancy having no functional significance, it is important in that it proves that there is no absolute specification of the cells on which Ia and Ib afferent fibres can establish functional synaptic connexion (cf. Eccles & Oscarsson, 1961).

When considering the function of the DSCT pathway in relaying information from muscles to the cerebellum, it has not been possible to interpret the various observations on the convergence of excitatory pathways in the Ia, Ib and II fibres from various muscles. The converging inhibitory influences are still more difficult to interpret. Detailed investigation of a far greater number of cells is necessary before attempting to develop postulates relating to the integration. However, it should be realized that there is very little or no convergence on most of the DSCT cells, which are thus specialized to relay information to the cerebellum from either the muscle-spindle or tendon-organ receptors of only one muscle or of closely related muscles. It is conceivable that many of the complex and apparently meaningless synaptic connexions are simply aberrancies arising in the circumstances of the original development which is controlled by relative rather than absolute specification of presynaptic to post-synaptic elements. In the functioning of the cerebellum the influence of these aberrancies would be equivalent merely to a background noise, upon which the meaningful signals from the specific pathways are superimposed. Communication from muscle receptors to the cerebellum thus qualifies as an example of the general communication problem involving signal to noise ratio.

SUMMARY

1. Intracellular recording from cells of the cat dorsal spinocerebellar tract has been employed in a detailed study of excitatory synaptic action and of the convergent patterns for the excitatory and inhibitory pathways of the Group Ia, Ib and II impulses from diverse muscles.

2. When spike discharges are produced by monosynaptic action of Group I a and I b volleys, there is a large rebuilding of the excitatory post-synaptic potential (EPSP) after the spike; and this may fire a second spike.

3. Usually there is progressive depolarization, causing the blockage of the spike and leaving EPSPs which have an initial large depolarization rapidly declining to a prolonged residuum; this residuum is due to the same prolonged transmitter action that rebuilt the EPSP after the spike.

4. I a and I b EPSPs were recorded at the steady state during repetitive activation at various frequencies. Relatively to very slow frequencies there was depression at moderate frequencies centring around 10/sec and a relative or even absolute potentiation at higher frequencies, up to 50–100/sec. Maximum rate of liberation of transmitter occurred above 300/sec.

5. Most DSCT cells were monosynaptically activated by either I a or I b volleys, but there were many examples of convergence of I a and I b impulses on the same cell. There was monosynaptic convergence of Group II impulses on each of these three categories of DSCT cells, though convergence on to I a activated cells predominated. There were many examples of convergence of I a and I b impulses from muscles of the most diverse function.

6. Inhibitory post-synaptic potentials produced by Group I a or I b muscle volleys always had a longer latency than the monosynaptic EPSPs, the difference being accountable to an interneurone on the pathway. Some DSCT cells resembled motoneurones in their pattern of I a and I b excitation and inhibition.

7. Preliminary experiments suggested that there was also presynaptic inhibition of the synaptic transmission in the DSCT pathway, just as with monosynaptic reflexes.

8. The convergent patterns of muscle afferent impulses on DSCT cells are compared with those of motoneurones and are considered in relation to problems of function, communication and development.

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THE FUNCTIONAL CHARACTERISTICS OF SINGLE CELLS IN THE CAUDAL PART OF THE SPINAL NUCLEUS OF THE TRIGEMINAL NERVE OF THE CAT

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The primary sensory nuclei of the trigeminal nerve are more closely grouped than those serving the skin of the trunk and limbs; and the spinal nucleus in particular is more accessible to investigation than the diffuse spinothalamic system with which it is held to be analogous. It stretches from the rostral medulla to the second cervical segment; and its afferent supply is made up of primary fibres of the trigeminal nerve which run caudally through the medulla and enter the nucleus along its lateral side. Sjöqvist (1938) showed that section of these afferent fibres in the caudal medulla, in man, caused facial analgesia with loss of thermal sense but preservation of tactile sense. The spinal nucleus might therefore be expected to have interesting properties, differing from those of the dorsal-column-lemniscal system, which have been much more extensively studied.

Our investigation was almost entirely limited to the caudal part of the spinal nucleus, which constitutes, in length, only about a third of the whole. A brief description of our results has been given earlier (Gordon, Landgren & Seed, 1960).

METHODS

All experiments were done with cats, anaesthetized with pentobarbitone sodium in an initial intraperitoneal dose of 38 mg/kg and subsequent intravenous doses of 5–10 mg. The animals were kept deep enough to prevent spontaneous movements or movements in response to stimulation. In the course of some experiments in which anaesthesia was steadily maintained for some hours, 8 mg of gallamine triethiodide was given intravenously to produce a neuromuscular block. The block lasted for 15 min or so, and artificial ventilation was used during this period.

The operation for exposing the caudal medulla and first cervical segment is described, with a photograph of this region, by Gordon & Paine (1960). The caudal part of the spinal trigeminal nucleus is partly overlaid by the cuneate nucleus, whose clear-cut surface markings were a useful guide in inserting electrodes. The cat's head was clamped in a frame attached to an animal-holding stand, using the internal auditory meatus, lower margins of the orbits, and hard palate as fixation points. The rod pressing upwards on the palate was

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small and slightly away from the mid line, so that as much of the mouth cavity as possible was free for applying stimuli. The neck was held by a clamp attached to the sides of one of the lower cervical vertebrae.

Recording. External records from single cells and fibres were made with glass micropipettes, filled with 3 M-KCl, and with a resistance at 50 c/s of 3·5–7 MΩ. Other details of recording technique are given by Gordon & Paine (1960).

Orthodromic stimulation. The receptive fields of all units responding to mechanical stimuli were explored in as much detail as possible, and those responding to movements of whiskers were always tested for directional sensitivity. A number of mechanoreceptive units were tested for thermal sensitivity, using a device by which a slowly-running jet of air at room temperature could be switched, without significant change of flow, to a jet of air at other temperatures ranging from about 0° to 60° C. These temperatures were measured thermoelectrically. Warm or cool water was sometimes introduced into the mouth cavity for the same purpose. A number of spontaneously discharging cells which did not respond to mechanical stimulation were also tested for thermal sensitivity. Some cells responded to steady pressure, and the thresholds of some of these were determined with a simple device consisting of a Perspex disk of known area acting against a spring calibrated in grams weight. Pressures were expressed in dyn/cm^2.

Pairs of electrically controlled mechanical stimulators (Gordon & Paine, 1960) were sometimes used to study interaction when precise timing of the stimuli was important. Measurements of minimum latency of response in some units were made by stimulating the skin electrically through pairs of steel needles with the points 1–2 mm apart.

Antidromic stimulation. In a number of experiments attempts were made to excite cells in the spinal trigeminal nucleus antidromically by stimulating through electrodes inserted in the region of the contralateral medial lemniscus in the caudal thalamus (Horsley–Clarke frontal plane 4–5). The technique for inserting the transverse row of five electrodes is described in detail by Gordon & Seed (1961).

Histological reconstruction. Records were kept of the rostro-caudal position (measured from the obex) and the transverse positions (measured from the mid line) of each electrode track, and measurements were made of the depth of each responding unit from the surface of the cord. In 6 of the 18 experiments attempts were made to locate the electrode tracks histologically and to reconstruct the positions at which responses had been found.

The technique of reconstruction was as follows. At the end of the experiments tracks were made on each side of the cord, caudal to the recording site, with a fine hypodermic needle loaded with Indian ink and inserted parallel to the plane of the electrode tracks. These ink tracks were a known distance apart; and measurement of this distance after fixation gave an indication of the shrinkage produced. In some experiments the last glass electrode used was broken off and its tip left *in situ*; and another similar electrode was inserted, at a known transverse distance, on the other side of the cord, and also broken off. These electrodes were removed after fixation and were found to have left clear tracks in the sections, which were easier to use than the ink tracks in estimating shrinkage: the position of the last recording site of the experiment was also very positively determined. The head and neck were then perfused, first with 0·85 % sodium chloride and then with 5 % formaldehyde-saline. Fixation was completed by immersion. The relevant tissue was removed, and the caudal surface trimmed until it was flush with the ink tracks. Serial paraffin sections, 25 μ thick, were then cut, parallel to the trimmed surface, through the relevant region. The sections were stained with thionine.

An attempt was then made to identify in the section some part of each electrode track (see Plate 1); and if any boubt existed about the identity of a track, that one was eliminated from the reconstruction. Tracings were then made from the sections showing the visible parts of each track, through which lines were then drawn along the presumed course of the rest of the track. A correction for shrinkage was then applied to the depth measurements

made during the experiments, and the calculated positions of the recorded responses were drawn in along the track line. Two alternative conventions were used in calculating these positions. In one, the surface reading made during the experiment was assumed to give the true surface: this assumption is not always justified, because of possible indentation of the surface by the electrode, obscuring of the surface by fibrin, and a variety of other local causes. In the other, the depth readings at the point where forelimb responses disappeared during entry of the electrode were assumed to correspond with the lower border of the cuneate nucleus. This second convention gave reconstructions in which the evidence from adjacent tracks fitted together much more consistently. Examples of the use of both conventions are given in Text-fig. 5.

RESULTS

Our results are derived from a study of 250 single neural units which responded to stimulating the facial hairs, or soft tissues, usually skin or mucous membrane, of the face and mouth cavity. Almost all of these were found in a region whose rostral limit lay 3 mm rostral, and caudal limit 11 mm caudal, to the obex. Fifty-five of these units we classify as fibres, on account of their monophasic positive spikes and negligible resting or injury discharge: there was good reason to believe that most of these were found in afferent tracts of primary trigeminal fibres (see e.g. Text-fig. 5(*c*)). The remaining units we classify as cells on account of their spikes being either diphasic or monophasic negative, with a resting discharge in the absence of stimulation, and high-frequency discharge upon injury by the electrode. When histological reconstructions were made, these latter units were found to be confined to nuclear regions. 'Fibre-type' and 'cell-type' action potentials are shown in Text-fig. 1 (*a'*) and (*c'*) respectively.

The cells could be divided into two main classes with strikingly different properties; and it is with this difference that we are mainly concerned in this paper. For immediate convenience we have called these two classes '*A*' and '*B*'.

'*A*' *cells*

These cells responded to ipsilateral 'tactile' stimuli; and it will be seen from our account of them that in general they closely resemble 'tactile' cells in other sensory nuclei. The vibrissal receptors of the face are known to be specialized, however (Fitzgerald, 1940); and we give a separate account of the cells responding to vibrissal stimulation, while including them in the '*A*' class.

General properties of '*A*' *cells.* These cells always responded at very low threshold to mechanical stimuli like bending hairs or touching the skin or mucous membrane lightly with a glass rod. Apart from vibrissal responses (see below), the discharge was almost always of a rapidly adapting kind. Seventeen out of the total of 140 '*A*' cells were tested with thermal stimuli, using streams of warm or cold air between the extreme limits of -2 and $+60°$ C: no evidence of thermal sensitivity was seen in any of these.

The majority of the '*A*' cells responded only to stimulation of the hairs

of the face. Their receptive areas were always well defined, ranging between 0·1 and 26 cm², with a mean at 3·7 cm². Two-thirds of these were of 1 cm² or less, and most of the small areas lay near the mouth or nose. Some of the larger receptive areas lay in the territory of more than one division of the trigeminal nerve.

About a fifth of the '*A*' cells responded to light touch on skin or mucous membrane, but not to touching hairs. The receptive areas of these ranged between 0·04 and 12 cm², with a mean at 0·41 cm². Within this group were 11 cells with receptive areas confined to the lips; and these areas were all very small (range 0·04–0·5 cm²; mean 0·11 cm²).

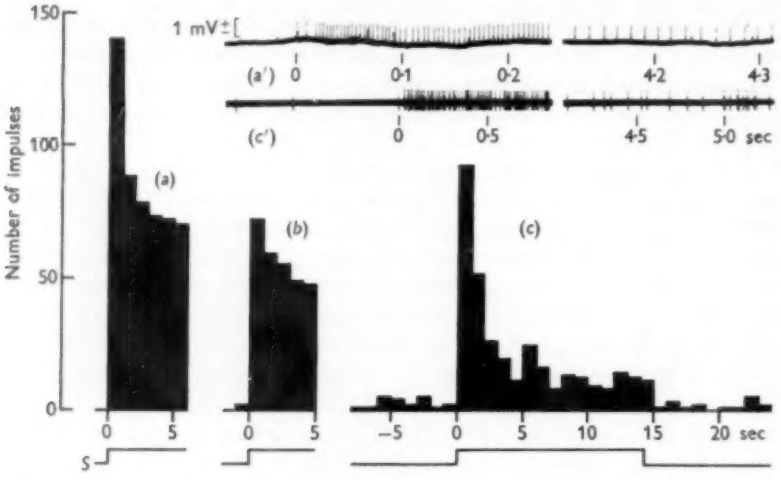
Cells and fibres responding to stimulation of vibrissae. The maxillary tactile hairs (or vibrissae) are thought to have a special sensory significance for the carnivores; and Fitzgerald (1940) showed that their nerve endings had the special properties of slow adaptation and directional sensitivity. We have recorded from a number of fibres, on the lateral side of the spinal nucleus, which responded only to vibrissal stimulation; and almost all of them showed these two special properties. Text-fig. 1 (*a*) and (*b*) shows the first few seconds of the responses to maintained steady displacement in two vibrissal fibres, with characteristically slow adaptation. Of the 20 fibres from which we recorded, 14 responded to stimulation of single vibrissae only. We have also recorded from a few fibres responding to stimulation of supraorbital tactile hairs. These showed the same special properties, and should probably be classified functionally with the maxillary vibrissae.

In the nucleus itself we recorded from a number of cells responding to vibrissal stimulation. The slow adaptation which was so characteristic of the fibres was uncommon among the cells, most of which adapted as quickly as tactile cells normally do, a fact which might depend on the presence of a general anaesthetic. Text-fig. 1 (*c*) shows a response in which adaptation was slower than in the majority of these cells. Clear-cut directional sensitivity also was much less common among cells than among fibres.

Only 10 out of 32 cells had receptive fields confined to one vibrissa alone. The number of vibrissae from which the remaining cells could be excited ranged from 2 to 12. This implies some spatial convergence of afferent fibres upon the cells; and this is borne out by our finding a small number of cells of rather large receptive area including both vibrissae and ordinary hair.

Latency of 'A' cell responses to electrical stimulation of the receptive surface. Latencies of the responses produced by electrical stimulation in the receptive area give an indication of the conduction time of the pathway. Latency shortens with increasing size of shock, because of temporal and

spatial summation in the nucleus; so that it is necessary to use shocks well above threshold and to find the minimum latency. Twelve '*A*' cells and 2 tactile fibres were tested in this way; and the minimum latencies, with an exception of 11 msec for one cell, lay between 1·7 and 4·2 msec. The



Text-fig. 1. Discharges in response to steadily maintained displacement of maxillary whiskers. Receptive area confined in each case to one whisker. Histograms show the number of impulses in each 1-sec period: the period of observation is shown by the length of the abscissa. The beginning and end of the stimulus are shown by upward and downward displacements respectively in line *S*.

(*a*) and (*b*) 'Fibre-type' responses in different units, recorded from a region 10 mm rostral to the obex and 6 mm from the mid line. A number of similar units were found in this region; and the electrode probably lay among afferent fibres of the Vth nerve. Part of the record from which (*a*) was drawn is shown in (*a'*), above; the spikes in both (*a*) and (*b*) were monophasic and positive.

(*c*) 'Cell-type' response, recorded from the spinal trigeminal nucleus 1·2 mm caudal to the obex and 3·75 mm lateral to the mid line (position verified histologically). Part of the record is shown in (*c'*), above: the spikes were diphasic (positive–negative).

Note the greater rate of adaptation and less regular discharge in (*c*) than in (*a*) or (*b*). All three units showed directional sensitivity to whisker displacement.

combined peripheral and central paths are approximately 80 mm long, but calculations of conduction velocity on this basis are hardly justifiable because of the existence of an unknown nuclear delay. These figures are useful mainly because of the comparison which will be made below between the latencies of '*A*' and '*B*' cells.

Inhibition of 'A' cells by stimuli outside the receptive area. Inhibition of sensory cells by stimulation outside, but usually near, their receptive areas

is now well known. It has been shown for cutaneous tactile cells in both the dorsal column nuclei (Amassian & de Vito, 1957; Gordon & Paine, 1960) and in the sensory cortex (Mountcastle, 1957). It is easily demonstrated by using a pair of electrically operated mechanical stimulators, one inside the receptive area (testing) and one outside it (conditioning). The time interval between these stimuli is controlled electronically.

A serious artifact can be introduced if these stimuli are applied close together on the skin; because the conditioning stimulus may spread and interact physically with the test stimulus in such a way as to reduce the effectiveness of the latter. A spurious 'inhibition' would then occur. We have used a control in our experiments by which we hoped to define this artifact quantitatively. Recordings were made from a number of single vibrissal *fibres* in the afferent tract, stimulating the appropriate vibrissa with the 'test' stimulator. The 'conditioning' stimulator was then applied to hairs or vibrissae at a number of known distances from the receptive vibrissa; and this stimulus was increased in size in an attempt to produce an 'inhibition'. It was assumed that all such 'inhibitions' were due to the physical artifact and that no genuine inhibitory interaction occurs between primary fibres. We were unable to produce the artifact with our largest conditioning stimulus (1·5 mm displacement of the hair 5 mm or more from the skin) when the two stimuli were 15 mm apart or more, and interaction rarely occurred when they were only 10 mm apart. With a separation of 5 mm interaction was seen in about half the tests made, and at strengths of conditioning stimulus small enough to make any confident distinction between genuine and spurious cell inhibitions impossible. We have therefore discarded all evidence from cells at spatial separations less than 10 mm; and have allowed a factor of safety in amplitude of conditioning stimulus of at least $\times 2$ when accepting any others as genuine.

Tests for inhibitory interaction were made for only 16 '*A*' cells, with a variety of receptive areas of 5 cm^2 or less, and 6 of these showed an undoubted inhibitory conditioning by stimuli applied at distances of 10–40 mm from the receptive area. Inhibition may well be commoner among these cells than our results show, because the region within 10 mm of the test stimulus, which we could not confidently use, may contain the most powerful inhibitory region.

Antidromic excitation of 'A' cells. Antidromic excitation of cells in the spinal trigeminal nucleus was attempted in some experiments, using stimulating electrodes in the region of the medial lemniscus of the opposite side at the level of the caudal thalamus. About 40 % (34 out of 82) of the '*A*' cells tested in this way gave responses which we regard as antidromic, using the criteria given by Gordon & Seed (1961). The latencies for the antidromic spike fell in the range 0·5–2·7 msec. One cell fired repetitively in response to the lemniscal shock, and had a longer latency than the rest (3·1 msec) and this may have been excited trans-synaptically.

It is clear, therefore, that a substantial number of '*A*' cells project to the contralateral thalamus in the lemniscal system.

Topographical arrangement of 'A' cells within the nucleus. It was said earlier that the '*A*' cells were found in the spinal nucleus itself. The evidence for this comes from the six experiments in which histological

reconstructions were made (see Methods); but in the other experiments also it was clear that these cells lay in a circumscribed region of the cross-section of the cord, separated by a 'silent band' 100–400 μ wide from the overlying cuneate nucleus. '*A*' cells with adjacent receptive areas were grouped together within the spinal nucleus, and an orderly topographical change in the receptive fields occurred along each recording track. Ventral movement usually gave a rostral shift in receptive field.

Cells with mandibular fields were found medially. Those with maxillary fields lay lateral and ventral to these; and those with ophthalmic fields lay lateral and ventral to the maxillary group. This oblique laminar arrangement of the cells belonging to the three divisions of the trigeminal area was found at all the rostro-caudal levels investigated; and agrees with the arrangement described by Darian-Smith & Mayday (1960). The tracings in Text-fig. 5 show some examples of the reconstructed electrode tracks from which this general arrangement was established.

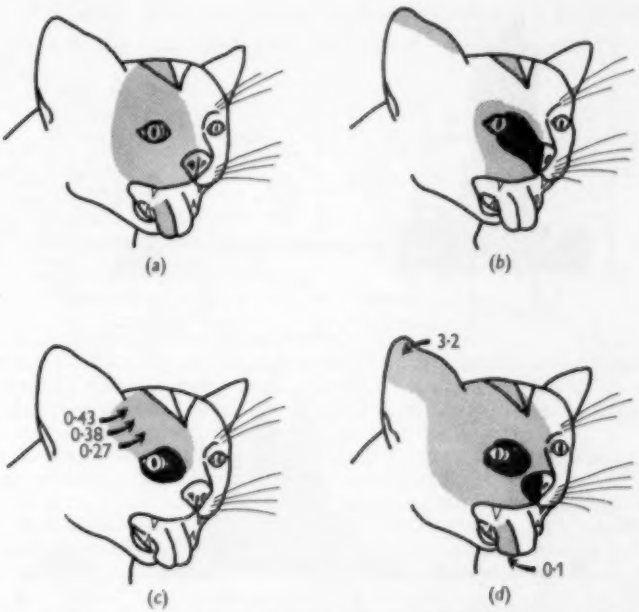
'B' cells

Twenty-one of the cells investigated formed a group ('*B*') with very distinctive properties, differing from the more numerous '*A*' cells in threshold, receptive area, and adaptation, and also in their anatomical position.

General properties of 'B' cells. The receptive areas of these cells were large, usually involving the territories of more than one of the divisions of the trigeminal nerve. The commonest type of receptive area occupied the maxillary and ophthalmic divisions; but some lay in the maxillary and mandibular divisions and a few included parts of all three divisions. The centre of the field usually lay in the region of the eye and side of the nose. The conjunctiva and nictitating membrane were often included, and sometimes the cornea as well. The field was not always in one continuous piece, and it was often possible to excite the cell from separate regions like the pinna or supraorbital periosteum, and sometimes from the tongue. When a cell was observed continuously for an hour or so the size of the receptive area was not always constant, but showed minor extensions or contractions. These changes were usually difficult to measure confidently, because the actual boundaries of the area could not be defined as precisely as with '*A*' cells. Four examples of the receptive areas of '*B*' cells are shown in Text-fig. 2. These areas were normally confined to the ipsilateral half of the face, but it will be seen that the example in Text-fig. 2(*d*) extends a little across the mid line.

The mechanical threshold for excitation of '*B*' cells was on the average much higher than for '*A*' cells. The contrast was particularly obvious in the peripheral parts of their receptive fields, where firm pressure with

a glass rod, light pinches or pinpricks were effective stimuli, but light touch was not. The centre of the field usually had a lower threshold (see Text-fig. 2), and here light touch, and even occasionally brushing hairs might be effective. In some experiments we tried to get estimates of the pressure thresholds in those parts of the receptive fields where pressure could be evenly applied; and it was usually found that this threshold increased



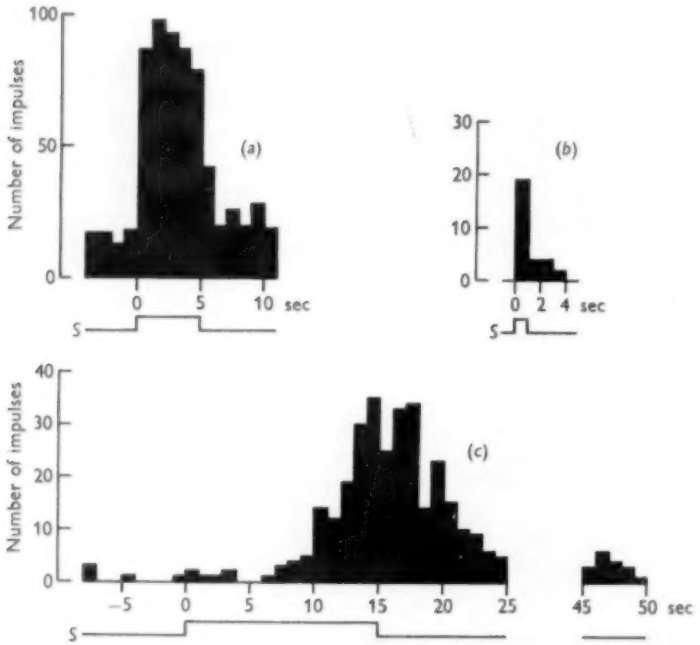
Text-fig. 2. Drawings to show the receptive areas of four '*B*' cells, in different animals. In each, the dotted area shows the extent of the receptive area, with the most sensitive part in black. The numbers in (*c*) and (*d*), multiplied by 10^6, give the thresholds to pressure in dyn/cm², in the parts of the receptive area where such measurements could be made satisfactorily.

The triangular area on either side of the mid line on the forehead represents the front end of a sagittal incision in the scalp, exposing the periosteum.

progressively towards the periphery (Text-fig. 2(*c*)). The measured thresholds in the periphery ranged between $0{\cdot}1 \times 10^6$ and $3{\cdot}2 \times 10^6$ dyn/cm². Eight of these cells were also tested with thermal stimuli, and seven responded, but only to extreme stimuli—over 50° C or less than 5° C. Such stimuli were felt as painful on the human arm after 15–20 sec.

The pattern of discharge of a '*B*' cell in response to a mechanical stimulus was always slowly-adapting, and the response often continued

for some seconds after the stimulus was withdrawn (Text-fig. 3(*a*) and (*b*); and Text-fig. 4(*d*)). This was also true of the response to extreme thermal stimuli (Text-fig. 3(*c*)). Irregular resting discharge at a low frequency, in the absence of stimulation, was very common among these cells. We did not find any obvious inhibition of this resting discharge when mechanical stimuli were given to various parts of the face on either side.



Text-fig. 3. Discharges of '*B*' cells. Histograms show the number of impulses in each 1-sec period: the period of observation is shown by the length of the abscissa. The beginning and end of the stimulus are shown by upward and downward displacements respectively in line *S*.

(*a*) Response of a '*B*' cell to pinching the upper eyelid.

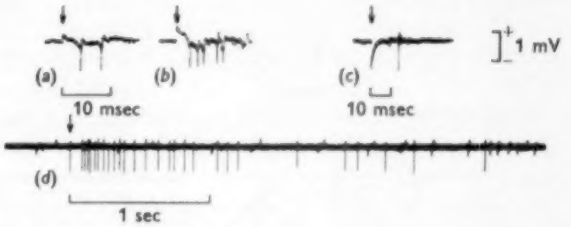
(*b*) Response of another '*B*' cell to pinching the pinna.

(*c*) Response of the same cell as in (*b*) to stimulating the medial angle of the eye with a jet of hot air at about 50° C. Part of the record between 25 and 45 sec has been omitted. Other responses of this cell are shown in Text-fig. 4, and its receptive area in Text-fig. 2(*b*). Its probable position is shown by the upper of the three crosses (×) in Text-fig. 5(*b*).

The high mechanical threshold of '*B*' cells, particularly when the stimuli were given to regions like the eye, nose and pinna, naturally raised the doubt whether the responses were secondary to reflex muscular

contraction. Seven typical '*B*' cells were tested both before and during neuromuscular block, however, without any alteration in the response being detected.

Latency of response of 'B' cells to electrical stimulation of the receptive surface. This latency was generally longer than that of '*A*' cells. In the cells tested the minimal latencies to stimulation in the most sensitive part of the receptive area ranged from 2·2 to 12·5 msec (6 out of 7 lying between 4·0 and 12·5 msec). When minimum values were obtained from the peripheral parts of the area they were three to six times greater (see Text-fig. 4 (*a–c*)).



Text-fig. 4. Discharges of a '*B*' cell.

(*a*) and (*b*). Two responses to single-pulse electrical stimuli to the edge of the upper eyelid near the medial angle. (*a*) Stimulus around threshold: latency 3·6 msec. (*b*) Stimulus approximately twice threshold: latency 2·2 msec. Latency did not shorten further with increase in stimulus.

(*c*) Responses of the same cell to single-pulse electrical stimulus to the supra-orbital periosteum. Maximal stimulus: latency 13·0 msec.

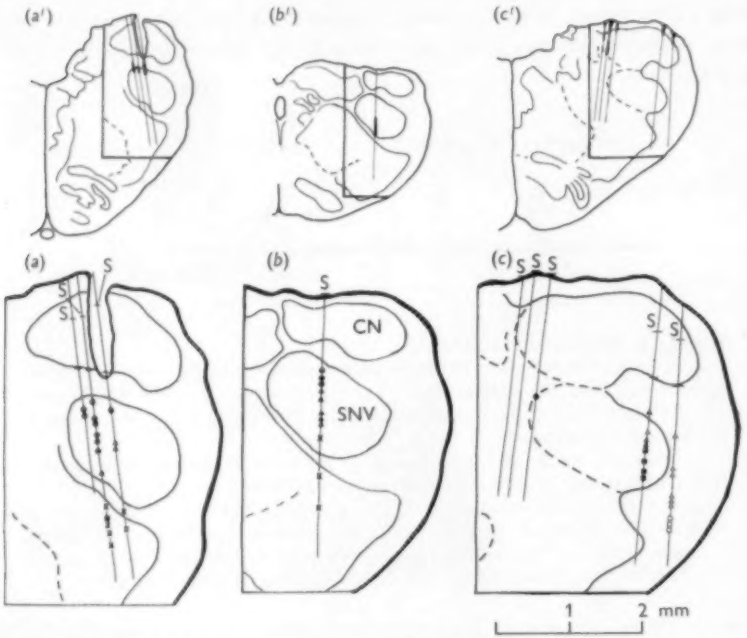
(*d*) Response of the same cell to pinching the pinna, the stimulus lasting about 1 sec. Spikes retouched. This response is also shown in Text-fig. 3(*b*). The receptive area of this cell is shown in Text-fig. 2(*b*); and its probable position by the upper of the three crosses (×) in Text-fig. 5(*b*).

Responses of 'B' cells to lemniscal stimulation. Of the 10 '*B*' cells tested one was fired antidromically by stimulating the region of the contralateral lemniscus; and five were fired in a way suggesting trans-synaptic excitation (see Gordon & Seed (1961) for our criteria of such excitation).

Positions from which 'B' responses were recorded. '*B*' cells were invariably found deep to the '*A*' cells, though it was unusual to find any 'silent band' separating the two. They were found at all the rostro-caudal levels investigated. Reconstructions of the electrode tracks show that some '*B*' cells may have been in the spinal trigeminal nucleus, very near its ventral border (Text-fig. 5(*b*)), but the errors possible in the reconstruction make a decision on this point unjustifiable. There is no doubt that a substantial number of these cells lay deep to the nucleus (Text-fig. 5(*a*)), in the region of the reticular nuclei which border the ventral and ventromedial aspects of the trigeminal nucleus at this level (see Pl. 1). Cells

responding in one or other phase of the respiratory cycle were often found deep to the '*B*' cells, and these were undoubtedly in the reticular nuclei.

Cells with similar properties ('*B*'-*type*). Seven cells were found which were similar in some but not in all their functional characteristics to the '*B*' cells described above. As the differences from the main group lay chiefly in the size and distribution of their receptive areas, it could be held



Text-fig. 5. Tracings of transverse sections of the spinal cord (all 1–2 mm rostral to the obex) from three animals, showing electrode tracks. (*a*), (*b*) and (*c*) are enlarged tracings of the areas framed in (*a'*), (*b'*) and (*c'*), above. The parts of each track definitely seen in the sections are shown as thickenings of the track lines in the upper tracings.

On some tracks a horizontal bar (S) is marked at the surface of the cord, indicating that in these tracks the depth positions of the recorded responses were calculated from the surface reading made during the experiment. In other tracks a bar is marked at the lower border of the cuneate nucleus, indicating that the positions were calculated from the point of disappearance of cuneate responses; the position of (S) in these tracks is likewise calculated, and does not coincide with the actual surface (see Methods).

The responses are classified as follows: ●, mandibular division of face, '*A*' cell; ▲, maxillary division, '*A*' cell; ■, ophthalmic division, '*A*' cell; ○, mandibular fibre; △, maxillary fibre; □, ophthalmic fibre; ×, '*B*' cell. *CN*, cuneate nucleus; *SNV*, spinal trigeminal nucleus.

that the distinction we are making here is only one of degree. There is clearly an extensive spatial convergence on to all the cells we have called '*B*', and the effectiveness of this convergence would be expected to vary with general levels of excitation and inhibition, and with the effects of anaesthesia upon these levels. There was no indication, however, that the differences in these cases depended on level of anaesthesia; and we feel that the distinction is worth making for immediate descriptive purposes.

Three of these cells had the general functional properties of '*B*' cells, and lay deep to the '*A*' cells in the cord; but they had fairly well-defined receptive areas, probably no more than 1 cm² in size, one on the pinna and two on the nose. The spontaneous activity of one of the latter was consistently inhibited by pressure on the ipsilateral side of the tongue; and this was the only occasion on which inhibition was seen in a cell with these general properties.

The other four cells differed from the main '*B*' group in one striking respect—that they could be excited by strong pressure outside the trigeminal area. They all had typical receptive fields in the face, extending in one case to the contralateral side, and they all responded to squeezing either of the forepaws. Two of them also responded to squeezing the hind paws, and one to similar stimulation of the trunk. All these cells were deep-lying; and one of them was found 0·8 mm deep to other typical '*B*' cells.

Other cells differing from 'A' cells. A number of cells responded to moderate pressure on the skin of the face, but closer investigation often suggested that the sense organs concerned lay deep in the substance of the lip, nose or gums, making any exact definition of their properties impossible. Five cells, however, had well-defined receptive areas of 2 cm² or less, and responded, three with slowly-adapting and two with rapidly-adapting discharge, to slight sustained pressure on the skin. The resting discharge of one of them was consistently inhibited by pressure on the skin of the same side of the face, or on the tongue. They differed from the tactile ('*A*') group in their somewhat higher mechanical threshold—of the order of 0·05–0·1 ($\times 10^6$) dyn/cm², in their failure to respond antidromically to contralateral lemniscal stimulation, and in lying deeper in the cord in a region where '*B*' cells might also be found. For lack of further information they remain an ill-defined group.

DISCUSSION

The part of the spinal nucleus which we have examined corresponds in rostro-caudal position to the part called by Meesen & Olszewski (1949), in the rabbit, 'nucleus tractus spinalis trigemini caudalis'. It also corresponds in position to the part of the spinal nucleus deafferented, in

man, by Sjöqvist's trigeminal tractotomy (Sjöqvist, 1938). The loss of pain and temperature sensation produced by this operation naturally led to the belief that there would be striking functional differences between the cells in the nucleus at this level and the cells of primary 'tactile' nuclei. Our findings suggest that in the cat, at any rate, this is not so: in their tactile responses, orderly topographical arrangement and lemniscal projection, the cells which we definitely localized to the spinal nucleus proper (our '*A*' cells) resemble those of other tactile nuclei such as the gracile and cuneate (see also Kuhn, 1949). Harrison & Corbin (1942) showed that tactile fibres run as far caudal as this in the spinal tract in the cat, and our results are a logical extension of theirs. Their paper gives a good account of the controversy which had continued for many years on this particular matter.

Our '*B*' cells form a group with remarkably consistent properties which contrast in many respects with those of the cells in typical tactile nuclei. These contrasting properties include a wide—but often discontinuous and fluctuating—receptive area, a threshold to mechanical stimulation which increases towards the periphery of the area, and a sensitivity to extreme thermal stimuli. They resemble closely the properties of the cells of the posterior group of thalamic nuclei, described by Poggio & Mountcastle (1960). They differ from the 'common carrier cells' found in the spinal dorsal horn by Wall (1960) in their larger receptive areas, and in their generally higher thresholds, particularly to thermal stimuli; though quantitative comparisons of this kind may not be justified, since our animals were anaesthetized with barbiturate, whereas Wall worked on unanaesthetized spinal animals. The nature of the convergence which gives '*B*' cells their sensitivity to different kinds of stimulus is not known. Local convergence of '*A*' cells upon them could only account for the relatively small tactile part of their sensitive range; and therefore they must receive either primary fibres with appropriately high thermal and mechanical thresholds from the spinal tract, or secondary fibres from a region unknown.

We have shown that at least a substantial number of '*B*' cells lay deep to the spinal nucleus, though superficial to cells with respiratory rhythms. This region seems to correspond to the 'nucleus reticularis ventralis' of Brodal (1957); though further work will be needed for its precise definition in terms of known anatomical structures. It lies ventrolateral to the reticular region in which Torvik (1956) found degeneration after trigeminal nerve section. It is of some interest that these '*B*' cells, excitable only from the ipsilateral face, and lying adjacent to the spinal trigeminal nucleus, lie in a nucleus of the reticular formation. They seem to belong to an organized facial afferent system rather than to show the spatially unspecific properties which are often attributed to sensory cells in these

nuclei; though this organization may merge spatially with other similar organizations, for a few ('*B*-type') cells were excitable from the limbs. Apart from one '*B*' cell fired antidromically from the contralateral medial lemniscus we have no knowledge of their efferent projections, and cannot therefore decide to what extent they belong to a rostrally-directed sensory system, or to a reflex system, or to both. Certainly these cells respond to stimuli in the range which one would call nociceptive; and it would be of great interest if their projections could be determined. The antidromic stimuli which we have used here may well have had access to only a limited part of the ascending trigeminal projections, about whose detailed anatomical organization there seems to be considerable uncertainty (see Rose & Mountcastle, 1959).

The complete absence of responses to small thermal stimuli in our experiments serves to emphasize that we have only examined a restricted region of the trigeminal nuclei. The thermal anaesthesia after Sjöqvist's tractotomy would lead one to expect such responses in the spinal nucleus; and by exclusion we should now expect that they would be found in its more rostral parts.

SUMMARY

1. The caudal part of the spinal nucleus of the trigeminal nerve was investigated, in anaesthetized cats, with extracellular micropipette electrodes, and responses recorded from single cells and fibres. A variety of mechanical and thermal stimuli were used to investigate the receptive areas and ranges of response. In some experiments attempts were also made to stimulate cells antidromically from the region of the contralateral medial lemniscus.

2. The majority of the cells (classed here as '*A*') had properties similar to those of 'tactile' cells in e.g. the dorsal column nuclei, responding to hair movement or light touch on skin or mucous membranes of the ipsilateral face. The responses were usually rapid in adaptation. Receptive areas ranged from 0·04 to 26 cm^2, the majority being rather small; and the smallest areas lay on hairy skin near the mouth or nose, or on the lips. Minimal latencies of response to electrical stimuli in the receptive area ranged from 1·7 to 4·2 msec. 40% of '*A*' cells responded antidromically to lemniscal stimulation.

3. Some '*A*' cells responded to stimulation of maxillary vibrissae or of the long supra-orbital hairs. Some but not all of these responses showed the slow adaptation and directional sensitivity characteristic of vibrissal sensory fibres.

4. Mechanical stimulation of neighbouring hairs was shown to inhibit the responses of some '*A*' cells.

5. Histological evidence shows that '*A*' cells lay in the spinal trigeminal nucleus. There was a systematic topographical arrangement in the transverse plane, mandibular cells lying medial and dorsal, ophthalmic lateral and ventral, with maxillary in between.

6. A smaller group of deeper-lying cells ('*B*') had larger receptive areas. These spread over a great part of the ipsilateral face, were often discontinuous, and tended to vary in extent with time. Thresholds for mechanical stimulation were often low in the centre of the area, but increased peripherally to values far exceeding those of '*A*' cells. Responses adapted slowly and often outlasted the stimulus. A number of '*B*' cells responded to extreme thermal stimuli. Minimal latencies for electrical skin stimulation lay between 2·2 and 12·5 msec. Only one '*B*' cell (out of ten tested) responded antidromically to lemniscal stimulation. A few cells, otherwise similar, could be excited by strong pressure on some or all of the limb extremities.

7. The anatomical location and possible functional significance of '*B*' cells is briefly discussed.

8. No cells were found which showed any appreciable response to thermal skin stimuli within the range 10–45° C.

We are very much indebted to Dr T. P. S. Powell and Dr W. M. Cowan for their generous help with histological reconstruction, for supplying the photomicrograph of Plate 1, and for much useful discussion.

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0·5 mm
SNV
VRN
CN
GN

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EXPLANATION OF PLATE

Plate 1. Photomicrograph from a transverse section of the spinal cord showing (in part) five electrode tracks, which are marked by arrows. Facial responses were recorded from the most lateral (i.e. right-hand) track, in which a '*B*' cell was found at a depth from the surface of approximately 1·2 mm (figure corrected for shrinkage in fixation). Tactile facial responses were recorded from the cellular region immediately superficial to this. CN, cuneate nucleus; GN, gracile nucleus; VRN, ventral reticular nucleus; SN*V*, spinal trigeminal nucleus. Section 25 μ thick: thionine stain.

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THE SPINAL PATHWAYS OF THE MYELINATED AND THE NON-MYELINATED AFFERENT NERVE FIBRES THAT MEDIATE REFLEX DILATATION OF THE PUPILS

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Stimulation of the skin or viscera of a cat anaesthetized with chloralose leads to a reflex dilatation of the pupils (Irving, McSwiney & Suffolk, 1937). After placing lesions in various parts of the spinal cord, Harper & McSwiney (1937) and Harris, Hodes & Magoun (1944) concluded that the afferent pathways for this pupillo-dilator reflex ascend within the lateral white columns of the cord. The reflex is brought about by the afferent impulses inhibiting the spontaneous activity in the parasympathetic division of the oculomotor nucleus, the cervical sympathetic fibres exerting little or no dilator action (Ury & Gellhorn, 1939).

Electronic stimulating and recording techniques have revealed that two groups of afferent fibres can contribute to the pupillo-dilator reflex, some of the afferents coming within the delta subdivision of group A, while other afferents are in group C and are therefore non-myelinated (Evans, 1957). Zotterman (1939) has suggested that 'fast' and 'slow' pain perception is mediated by afferents in the A-delta and C groups respectively. Some observations, both on man and on experimental animals, suggest that nerve impulses associated with pain ascend in more than one region of the spinal cord (Lalonde & Poirier, 1959; see also White & Sweet, 1955). It therefore seemed advisable to investigate the individual spinal pathways of the A-delta and C groups of afferents, using reflex pupillo-dilatation in the cat.

The present paper gives details of the techniques used in establishing the existence of the two classes of pupillo-dilator afferent nerve fibres, and also gives the results obtained after placing lesions in the spinal cords of cats. No major differences have been found between the spinal projections of the A-delta group and the C group of the pupillo-dilator afferents. Both groups ascend diffusely within the regions of the cord indicated by Harper & McSwiney (1937).

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METHODS

Cats weighing between 2·1 and 5·9 kg were anaesthetized with chloralose, an initial dose of 70 mg/kg given intravenously. Additional amounts were given later if necessary. In some cases small quantities of pentobarbitone (5–10 mg/kg) were also given, to depress the general reflex excitability of the preparation.

After tracheotomy the cervical sympathetic trunk was divided on one or both sides near the level of the cricoid cartilage. When testing for the pupillo-dilator reflex the pupil of the sympathectomized eye was observed through a low-power microscope. The width of the pupil could be measured to within 0·05 mm by means of an eyepiece graticule.

Hind limb nerves were dissected out and prepared for stimulation. Usually the saphenous nerves were employed, but occasionally the sural or lateral popliteal nerves in each hind limb were exposed. The nerves were divided and the central end freed from tissue for 1–2 cm, care being taken to preserve the blood supply as far as possible. In some experiments the nerve was stimulated through buried electrodes, so the freed central end was ligated and drawn into a Perspex tube containing the platinum-wire electrodes, and the tube was then sewn in place before closing the wound over it. However, in other experiments a different technique was adopted, in order to record the compound action potential evoked by the stimulation. In these experiments a longer length of nerve was exposed and was freed from surrounding tissues at two places separated from each other by a few centimetres. The central portion of cleaned nerve was laid in continuity upon a pair of platinum-wire stimulating electrodes. The peripheral portion was divided and laid upon a similar pair of recording electrodes, and the end of the nerve was crushed so as to obtain monophasic records. With this arrangement it was possible to determine the appropriate stimulus strength needed to excite the various elevations in the compound action potential. When doing this, the stimulus cathode was the more distal of the stimulus pair, and conduction velocities were calculated from the stimulus response interval and the conduction distance *in situ* from cathode to recording electrodes. Having determined the appropriate stimulus strengths needed to excite the various elevations in the compound action potential, the leads to the stimulating electrodes were reversed so as to place the cathode central, and the same stimulus strengths were used to send corresponding impulses into the spinal cord.

A conventional RC-coupled amplifier with a cathode-follower input probe was used in displaying the nerve action potentials on cathode-ray oscilloscopes. The stimulator was set to deliver rectangular pulses of 250 μsec duration at a repetition rate of 5/sec. The pulses were applied to the nerve from a radio frequency isolation unit that allowed stimulus strengths of up to 40 V to be used. A pulse duration of 250 μsec was chosen because this was sufficiently long to allow stimulation of C fibres within the maximum available voltage, while avoiding the re-excitation of A fibres that occurs at the end of longer pulse durations. A repetition rate of 5/sec was chosen because at this frequency the reflex pupillary response developed reasonably rapidly and would be maintained at a steady dilatation for at least 30 sec. When testing the reflex pupillary dilatation the train of stimulus pulses was usually applied for 30 sec, with about 5 min rest between successive periods of stimulation.

In some experiments it was necessary to paralyse the anaesthetized cat in order to prevent reflex movement from interfering with the recording of action potentials. D-Tubocurarine was unsuitable because it produced a considerable pupillary dilatation. Decamethonium iodide or gallamine triethiodide were found to be suitable: fully paralysing doses could be given without interfering with the reflex pupillary dilatation. Decamethonium iodide (Burroughs Wellcome) was used most commonly, made up at a concentration of 100 μg/ml. in NaCl solution 0·9 g/100 ml. It was given intravenously in amounts sufficient to maintain adequate paralysis, and artificial ventilation was applied for the duration of the paralysis.

In some experiments adrenaline was given intravenously. Adrenalin chloride solution (Parke, Davis) was diluted to the appropriate concentration with 0·9 % NaCl solution.

In several cats lesions were made in various parts of the spinal cord. Semisections were made by entering a fine tenotomy knife into the cord along the desired line of section and then cutting down to the knife with scissors or a narrow fragment of a razor blade. After a lesion had been made the preparation was rested for at least half an hour before testing the pupillo-dilator responses to nerve stimulation. Artificial ventilation was given whenever it was necessary. At the end of the experiment the part of the cord containing the lesion was fixed and serial paraffin sections were cut and stained. From these sections diagrams were prepared showing the total extent of the lesions produced by the cutting plus any subsequent haemorrhage.

RESULTS

The classification of pupillo-dilator afferents

Soon after anaesthetizing the cats with chloralose the pupils became constricted, as is usual when using this anaesthetic. By the time the dissection of the hind limb nerves had been completed the pupils had a

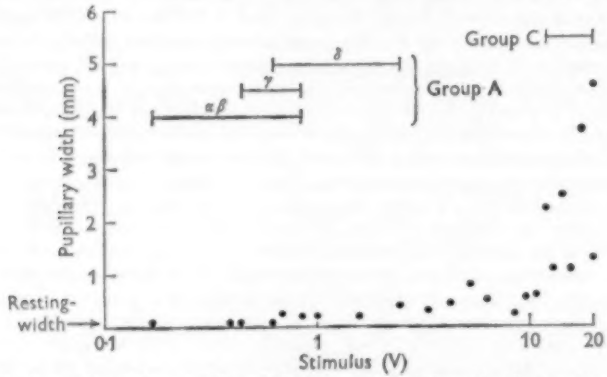

Fig. 1. A graph of the pupillary dilatation evoked by stimulating the right saphenous nerve at various stimulus strengths. Obtained from an experiment on a cat anaesthetized with chloralose 86 mg/kg plus pentobarbitone 6 mg/kg. Ordinate: width of pupil during stimulation; the resting width was 0·1 mm. Abscissa: voltage of the stimulus shocks, log. scale. The horizontal bars indicate the voltages (from threshold to maximal) during which the different elevations appeared in the compound action potential.

resting width that was almost always less than 1 mm. If one of the exposed nerves was stimulated at 5 pulses/sec for about 30 sec both the pupils dilated equally and soon reached a more or less constant width. This width depended upon the strength of the stimulus shocks. At any stimulus strength the reflex pupillo-dilatation could be correlated with the elevations evoked in the compound action potential of the nerve being stimulated (Evans, 1957).

In Fig. 1 pupillary dilatation is plotted against the voltage of the stimulating shocks that were applied to the saphenous nerve of a cat. The elevations that were evoked in the saphenous nerve compound

action potential by the various voltages are also indicated above the graph. The large alpha–beta elevation of Group A appeared at a threshold of 0·17 V and was fully developed at 0·85 V, and in this experiment the elevation travelled along the nerve at a velocity of 35–70 m/sec. The small gamma elevation was also fully developed at 0·85 V and had a conduction velocity of 30 m/sec. The delta elevation appeared at a threshold of 0·65 V and was fully developed at 2·5 V. This elevation contained components with conduction velocities of 15–25m/sec. The Group C elevation appeared at a threshold of 12 V and was well developed at 20 V, containing components with conduction velocities in the range 0·7–1·0 m/sec.

No reflex dilatation of the pupil resulted from stimulation of the nerve with shocks below the A-delta threshold. For instance, at a stimulus strength of 0·6 V no dilatation was seen, although the alpha, beta and gamma elevations were present.

When the stimulus was above the A-delta threshold, a small reflex dilatation of the pupil was seen, and with strengths between 0·65 and 2·5 V the pupils dilated 0·1–0·4 mm wider than their resting width of 0·1 mm. Stronger stimuli caused dilatations that were only slightly greater, until the strength was above the threshold of Group C. Above 12 V the Group C elevation was present and stimulation evoked a pupillary dilatation that was always more than that seen with stimuli weaker than 12 V; at 20 V a dilatation of 4·6 mm was observed.

The threshold voltage at which the various elevations appeared varied somewhat in different preparations, but there was always the clear difference between a modest dilatation produced by stimulation of the afferents in the A-delta group and a great dilatation produced when the stimulus strength was sufficient also to excite the C group of afferents.

Under the usual experimental conditions, when using stimulus pulses of 250 μsec and an electrode separation of 4–7 mm, the voltage required to stimulate all the Group A fibres was never greater than 4 V. On the other hand, the threshold of the Group C fibres was never less than 8 V. Consequently, when the physical arrangements in the later experiments required the use of buried electrodes to stimulate the saphenous nerves, this clear difference in thresholds was made use of in the absence of monitored action potentials. In these later experiments a stimulus of 4·0 V was used to excite the Group A fibres only, and 30–40 V was used to excite both the A and C groups. Although the elevations of the compound action potential could not be checked when using the buried electrodes, it is thought that this arbitrary setting of stimulus voltages was permissible because it was still possible to check the voltage thresholds for reflex movement and pupillary dilatation in these preparations, and these thresholds were always within the limits that had been observed in

the earlier experiments when the evoked compound action potential had been monitored.

Although the pupillo-dilatation evoked by simultaneously stimulating the A and C groups of afferents was always greater than that evoked by the A-delta afferents alone, the dilatation could not be used as a quantitative measure of the numbers of afferent fibres stimulated. This is shown in Table 1. In the experiment from which the figures are taken, the Group A-delta afferents were stimulated first in the left saphenous nerve and then in the right nerve, and the resulting pupillo-dilatations were almost equal. When the stimulus was given simultaneously to both nerves, the reflex pupillo-dilatation was only about 30 % greater, showing occlusion between the two afferent pathways. The pupillo-dilator reflex mechanism was itself capable of giving a response greater than the sum of the separate Group A-delta responses, as is shown by the large dilatation evoked when the stimulus strength was increased to activate both the A and C fibres in either the left or the right saphenous nerves.

TABLE 1. Reflex dilatation of the pupils evoked by stimulation of the saphenous nerves, either separately or simultaneously, at moderate and at high stimulus strengths. Cat anaesthetized with chloralose 70 mg/kg

Fibre size activated	Side of stimulation	Resultant dilatation (mm)
A-delta	Left	1·50
A-delta	Right	1·55
A-delta	Both	2·00
A and C	Left	4·10
A and C	Right	3·70

The greater pupillo-dilatation evoked by the strong stimuli could not have been due to repetitive firing of the A-delta afferents. This was shown by varying the frequency of stimuli which were slightly supra-maximal for the A-delta fibres. Although the frequency of the stimuli affected the rapidity with which the pupil dilated, the final width of the pupil was almost independent of the frequency, except that at frequencies greater than 20/sec the reflex became fatigued rapidly and the final width was only maintained briefly.

The dilatation of the pupils was not caused by reflexly induced activity in the sympathetic nervous system. Direct sympathetic effects were prevented by section of the cervical sympathetic trunk at the beginning of all experiments. Indirect effects, due to the release of adrenaline, could not account for the large dilatations seen.

An experiment from which the tracing shown in Fig. 2 was taken proved that maximal stimulation of a saphenous nerve did not result in sufficient adrenaline being released to affect the pupils. In most cats, including the one used in this experiment, it was found that 100 μg of adrenaline given

intravenously during 30 sec could produce a dilatation of the pupils comparable to that evoked by maximal stimulation of a saphenous nerve. About 10 μg were necessary to produce a detectable dilatation. Upon recording the contractions of the acutely denervated nictitating membrane shown in Fig. 2 it was possible to detect 1–2 μg of adrenaline injected intravenously during 30 sec. During maximal stimulation of either saphenous nerve the nictitating membrane did not indicate the release into the circulation of a significant quantity of adrenaline.

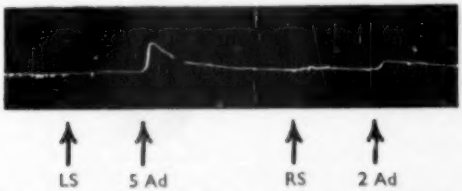

Fig. 2. Record of the contractions of the acutely denervated nictitating membrane of a cat anaesthetized with chloralose 71 mg/kg plus pentobarbitone 4·75 mg/kg. At LS the left saphenous nerve was stimulated at 5 shocks/sec for 30 sec at a voltage sufficient to activate both A and C groups. At 5 Ad 5μg of adrenaline was given intravenously for 30 sec. At RS the right saphenous nerve was stimulated strongly. At 2 Ad 2 μg of adrenaline was given in 30 sec.

Interruption of the ascending spinal pathways

The fibre groups that carried afferents capable of evoking pupillo-dilatation having been established, some experiments were done to determine the approximate location of the spinal projections of these afferents. Measurements were made of the pupillo-dilatations evoked by stimulating the Group A afferents, and of the greater dilatations evoked when the stimulus was strong enough also to excite the Group C afferents. Partial sections of the spinal cord were then carried out and the pupillo-dilatations were again tested and measured.

The lesions were usually placed in the 2nd or 3rd cervical segments; in a few preparations the lesions were placed in lower thoracic or upper lumbar segments. Both pupils continued to react equally to stimuli applied unilaterally. The stimuli were usually applied to the saphenous nerves. In a few preparations the sural nerves were utilized instead, and gave similar results. In four preparations the lateral popliteal nerves, containing muscle and skin afferents, were stimulated. These also gave results similar to those obtained with stimulation of purely cutaneous afferents in the saphenous nerves.

Lateral semisection of the cord. The reflex pupillo-dilatation evoked by stimulation of either saphenous nerve was never abolished but usually

slightly reduced after section of one half of the spinal cord in the cervical region. This applied both to the modest dilatation evoked by the A-delta afferents and to the greater dilatation evoked when the Group C afferents were also stimulated. There was no evidence that either the A-delta or the C afferents projected along pathways confined to one side of the spinal cord. In several experiments semisection resulted in the approximately equal reduction of the dilatation produced by stimulating the nerves in either hind limb, indicating that the pupillo-dilator afferents in the A-delta and C groups project as much in ipsilateral as in contralateral spinal pathways.

	Cord intact		Hemisection	
	L	*R*	*L*	*R*
A	0·8–1·1	0·2–0·4	0·7–0·8	1·1
A+C	2·0	2·1	1·3	1·4

Fig. 3. The results of placing a semisection in the second cervical segment of the spinal cord of a cat anaesthetized with chloralose 91 mg/kg plus pentobarbitone 4·75 mg/kg. The upper row of figures indicates the pupillary dilatation in mm evoked by stimulating Group A-delta fibres in the left (*L*) or right (*R*) saphenous nerves. The lower figures indicate the dilatation evoked when the stimulus strength was increased to stimulate Group C fibres. The figures in the left-hand block were obtained with the cord intact, those in the right-hand block were obtained ½ hr after placing the lesion illustrated above them.

The effects of section of the left side of the spinal cord in the 2nd cervical segment of one preparation are illustrated in Fig. 3 and the accompanying table. The magnitude of the dilatation evoked by stimulating the Group A fibres in the left saphenous nerve is scarcely affected by the lesion. The dilatation evoked from the Group A fibres in the right saphenous nerve was in fact greater after the semisection, but this is probably fortuitous because it was not seen in other similar experiments. The dilator responses to combined stimulation of Groups A and C in either nerve were reduced by an equal amount after the semisection.

In a few experiments two semisections were made at different levels, one on each side of the spinal cord. The upper semisection was made in the 2nd or 3rd cervical segment. The lower semisection was made in a segment ranging from the 12th thoracic to the 1st lumbar; in two cases this lesion was made aseptically 7 or 10 days before the experiment. In those experiments in which the subsequent histological examination showed that each

lesion was a complete semisection, the combined effect of the two opposing semisections was to abolish all pupillo-dilatation when the hind limb nerves were stimulated. There is thus no evidence that these pupillo-dilator afferents, either from the delta subdivision of Group A or from Group C, can cross within the thoracic or lower cervical segments of the cord. In two preparations, however, the semisections were incomplete and spared some of the fibres in the ventral part of the cord. In these two experiments hind limb nerve stimulation continued to evoke a slight pupillo-dilatation.

	Cord intact		First lesion		Second lesion	
	L	*R*	*L*	*R*	*L*	*R*
A	1·0–1·7	1·3–1·9	1·5	1·6	1·9	2·1
A+C	5·5	5·0	4·1	3·7	3·0	2·5

Fig. 4. The results of placing lesions in the dorsal part of the third cervical segment of the spinal cord of a cat anaesthetized with chloralose 70 mg/kg. The arrangement of the figures is the same as in Fig. 3, and they were obtained ½ hr after placing the lesions illustrated above them.

Section of the dorsal parts of the cord. When the dorsal columns were divided the evoked pupillo-dilatation was unchanged. Even when a lesion cut the dorsal one third of the cord and destroyed Lissauer's tracts and parts of the dorsal grey horns there was no significant reduction in the dilatation. More extensive lesions, that also involved parts of the lateral columns, did bring about a reduction in the pupillo-dilatation, especially that evoked by the Group C afferents.

This is illustrated in Fig. 4. A lesion in the dorsal one third of the 3rd cervical segment destroyed Lissauer's tracts, part of the dorsal columns and the greater part of the dorsal spinocerebellar tracts. After placing this lesion the pupillo-dilatations evoked by the Group A fibres were unchanged, while the dilatations evoked by the Group C fibres were slightly diminished (4*A*). A second lesion was then made 3 mm rostral to the first lesion, and this second lesion destroyed the dorsal half of the cord at this level (4*B*). Even this lesion did not bring about a reduction in the pupillo-dilatation evoked by stimulation of Group A fibres, but the responses to Group C stimulation suffered a further reduction.

In another similar experiment a more extensive lesion spared only the

ventral one third of the cord at the 3rd cervical level. Again the pupillo-dilatation evoked by stimulation of Group A fibres was scarcely affected, but the additional dilatation following C fibre stimulation was almost abolished.

Section of the ventral parts of the cord. The finding that the pupillary responses were not reduced by section of the dorsal one third of the cord, including Lissauer's tract, did not exclude the possibility that a small number of fibres in this region of the cord might be able to conduct impulses that would bring about a pupillo-dilatation. However, this possibility is excluded by the results obtained after placing the lesions illustrated in Fig. 5. The first lesion (5*A*) cut the ventral half of the cord and had only a small effect on the pupillo-dilatation. The second lesions

	Cord intact		First lesion		Second lesions	
	L	*R*	*L*	*R*	*L*	*R*
A	5·2–7·0	2·5–4·3	3·6	5·5	0	0
A+C	5·8	5·9	4·8	4·8	0	0

Fig. 5. *A*, the result of placing a lesion in the ventral half of the second cervical segment of the spinal cord of a cat anaesthetized with chloralose 110 mg/kg. *B*, the result of making two additional lesions in the lateral columns. The arrangement of the figures is the same as in Fig. 3 and they were obtained ¾ hr after placing the lesions illustrated above them.

(5*B*) extended the severed area so as to include most of the lateral columns and abolished the responses, although subsequent histological examination showed that the dorsal horns and Lissauer's tract had not been cut.

Individual variations in the ventral column pathway. The extent to which the pupillo-dilator afferents projected in the ventral white columns appeared to be variable. In some experiments, such as that illustrated by Fig. 4, the responses, at least to A-delta stimulation, were only slightly affected by lesions that interrupted a large part of the lateral columns. The lesions in four other experiments are illustrated in Fig. 6. Lesions *A* and *B*, on the left of Fig. 6, resulted in a reduced pupillo-dilatation, with the responses to Group C stimulation reduced more than the responses to Group A. On the other hand, the similar lesions *C* and *D* on the right of Fig. 6 abolished all reflex pupillo-dilatation.

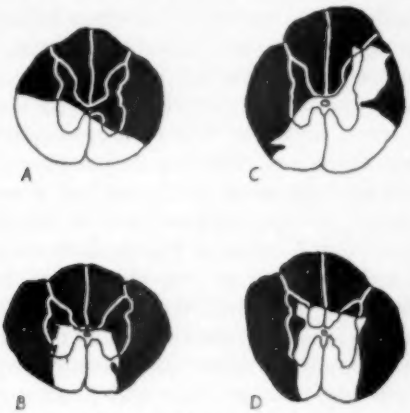

Fig. 6. Composite diagrams showing the cross-sectional area of destruction in the second or third cervical segments of the spinal cord, obtained from four different experiments. Each diagram is made up from the superimposition of two or more lesions. In the experiments illustrated by *A* and *B*, on the left, the lesions only succeeded in reducing the pupillary dilatation evoked by stimulation of the saphenous nerve. In the experiments illustrated by *C* and *D*, on the right, all the pupillary dilatation evoked by saphenous stimulation was abolished. In the experiment illustrated by *C* the cat was deeply anaesthetized with chloralose 140 mg/kg and only the right side saphenous nerve was stimulated. In the other three experiments the cats were anaesthetized with chloralose 70–90 mg/kg and both saphenous nerves were tested.

DISCUSSION

In spite of a considerable amount of work devoted to the afferent projections in the spinal cord of man and animals, including the investigation of the pathways which carry impulses leading to the sensation of pain, there is surprisingly little information about the central projections from the non-myelinated afferent nerve fibres. The results of the present experiments show that reflex pupillo-dilatation is evoked by afferents included in the finely myelinated A-delta and in the non-myelinated C groups, whereas the large-diameter myelinated afferents in the alpha-beta-gamma subdivisions of Group A play no part in the reflex dilatation of the pupil to sensory stimulation. Furthermore, it has been found after placing lesions in the spinal cord, that both the A-delta and the C groups of pupillo-dilator afferents ascend in the lateral columns. These results agree with those of Harper & McSwiney (1937), who placed lesions in the lower cervical segments of cat spinal cord and excited afferents in the splanchnic or intercostal nerves by Faradic stimulation. As they gave no details of their stimulus strengths one cannot estimate the threshold of the afferents which they stimulated. The projections of both the A-delta and C groups are

bilateral and are as diffuse as Harper & McSwiney indicated. The overlap between the two groups is so extensive that only minor differences can be detected between their spinal pathways.

In the spinal pathways of both A-delta and Group C afferents the ipsilateral projection seems to be as well developed as the contralateral projection. The only significant difference between the two groups is the tendency for the Group C projection to lie slightly dorsal to the A-delta projection, as is shown by the relatively greater effect of dorsal semisections upon the Group C evoked pupillo-dilatation. However, the pupillo-dilatation technique cannot be relied upon to yield accurate quantitative results, and even with the dorsal two thirds of the cord severed the increased dilatation evoked by Group C stimulation was often obtained, although to a reduced degree. Downman & Evans (1957) found that the A-delta afferents in splanchnic and peroneal nerves projected diffusely and bilaterally in the ventrolateral columns. Little evoked electrical activity was found in the dorsal part of the lateral columns. This would seem to be at variance with the present finding that ventral semisection did not significantly reduce the dilatation evoked by stimulation of the A-delta group in the saphenous nerve, but it would accord with the concept of a non-myelinated projection in the dorsal part of the lateral columns, a region known to be rich in non-myelinated fibres (Ranson, 1913). Such non-myelinated fibres would yield potentials too small to be detected by the micro-electrode techniques of Downman & Evans.

It is interesting to compare the spinal projections of the afferents which evoke pupillo-dilatation with the distribution of afferent pathways that mediate other nociceptive reflexes or pain perception. It is well known that weak Faradic stimulation of a sensory nerve evokes a fall in blood pressure in anaesthetized cats, whereas strong stimulation evokes a pressor response. The stimulus thresholds quoted by Martin & Lacey (1914) show that the depressor response is evoked by stimulus strengths similar to those that evoke the flexor reflex, thus indicating that the afferents are small-diameter myelinated fibres, probably in the A-gamma or delta subgroups. Ranson & von Hess (1915) found that the depressor response was dependent upon an ascending pathway in the lateral columns of cat spinal cord, and because the pathway was predominantly crossed they suggested that the afferents which evoke the depressor response might be identical with those subserving pain sensation. They took this view in spite of their own demonstration that the ascending paths subserving pain were equally crossed and uncrossed in cat spinal cord.

Ranson & von Hess (1915) found that the pressor response evoked by strong Faradic stimulation was dependent upon an ascending pathway in Lissauer's tract, lying between the dorsal root entry and the dorsal grey

horn. This projection was bilateral in cat spinal cord. They also showed that this pressor pathway in Lissauer's tract is not an important pathway for the transmission of impulses that evoke pain, and the present experiments have shown that Lissauer's tract does not transmit impulses that evoke pupillo-dilatation.

The tracts that carry impulses that lead to pain sensation appear to be diffuse and bilateral in cat spinal cord (Ranson & von Hess, 1915) although Kennard (1954) considers that the main pathway lies in the dorsal part of the lateral columns. Lalonde & Poirier (1959) also think that deep pain sensation in monkeys is dependent upon a pathway in the dorsolateral quadrant near the lateral corticospinal tract. Karplus & Kreidl (1914) found that cats reacted as though feeling pain when noxious stimuli were applied to tail or hind limbs, soon after recovering from the anaesthetic under which semisections had been made in upper cervical and lower thoracic segments on opposite sides of the cord. Their semisections appear to have been accurate and complete, and would be expected to interrupt all the long ascending pathways from the hind quarters. However, the present experiments have shown no evidence that the afferents which evoke pupillo-dilatation crossed more than once within the spinal cord. The only anatomical difference between the present experiments and those of Karplus & Kreidl is that they placed the cervical semisection very high, usually in the 1st cervical segment approached through the atlanto-occipital membrane. This may be significant, as Morin (1955) has demonstrated an extralemniscal decussation of cutaneous impulses in the nucleus cervicalis lateralis, in the 1st and 2nd cervical segments of cat spinal cord. It is possible that Karplus & Kreidl might not have obtained the reactions indicative of pain had they placed their upper semisection caudal to the nucleus cervicalis lateralis; they mention placing lesions in the 3rd cervical segments of a few cats but they do not discuss the results seen in these cases.

It is difficult to say whether there is a close relationship between the afferents that evoke reflex pupillo-dilatation and those that subserve pain. There are several similarities in the two systems, but there are also some differences that are not yet fully explained. In view of the many uncertainties regarding the location of the 'pain-conducting' pathways in cat spinal cord, which appears to differ from that of monkey and man, the relationship between these two systems must remain an open question.

SUMMARY

1. Stimulation of afferent nerves leads to reflex pupillary dilatation in cats anaesthetized with chloralose. The degree of dilatation depends upon the strength of the stimulus.

2. As the stimulus strength is increased the dilatation goes through two fairly well defined stages. Weak stimuli that excite only the largest myelinated fibres do not produce any dilatation. Moderately strong stimuli that also excite small myelinated fibres produce a moderate dilatation. Very strong stimuli that also excite non-myelinated fibres produce a large dilatation.

3. The two stages of pupillary dilatation evoked by moderate and by very strong stimulation have been measured before and after making lesions in various parts of the spinal cord.

4. The conclusion is drawn that the finely myelinated and the non-myelinated afferents responsible for the reflex pupillary dilatation both project bilaterally up the spinal cord. The pathways are diffusely distributed throughout the lateral and ventral columns. There is a tendency for the pathway from non-myelinated afferents to lie more dorsally than the pathway from finely myelinated afferents, but both pathways intermingle to a great extent.

5. The findings are discussed in the light of our present knowledge concerning the spinal pathways subserving pain and the nociceptive reflexes.

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TRANSFER OF IRON FROM THE PREGNANT AND LACTATING RAT TO FOETUS AND YOUNG

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In the pregnant rat the transfer of iron to placentae and foetuses is accompanied by depletion of iron stores (Smythe & Miller, 1929; Steenbock, Semb & Van Donk, 1936; Widdowson & McCance, 1948; Nylander, 1953). During lactation, although there is a further transfer of iron, as shown by the considerable increase in total body iron of the suckling (Smythe & Miller, 1929; Alt, 1938; Lintzel, Rechenberger & Schairer, 1944; Huggett & Widdas, 1950; McCance & Widdowson, 1951) the maternal stores show only a slight further decrease (Widdowson & McCance, 1948). Only Nylander (1953) appears to have correlated maternal and foetal changes in the same group of experiments. He also studied the effects of maternal iron depletion produced by repeated haemorrhage during pregnancy, but the effects of iron loading do not appear to have been reported.

The aims of the present experiments were (1) to correlate the changes in maternal storage and transport of iron during pregnancy and lactation with the accumulation of iron in foetuses and placentae and in the suckling and (2) to determine the effects of increased and decreased iron stores at the time of conception respectively on transfer of iron from mother to offspring during pregnancy and lactation in the rat.

METHODS

General plan of the experiment. Three groups of rats were used, each consisting of thirty-six females aged 18–22 weeks and weighing 170–240 g. Thirty animals from each group were mated. The other six rats were not mated and were used to provide non-pregnant values for comparison with pregnant and lactating animals. All the rats were fed from the time of weaning and during pregnancy and lactation on the same standard laboratory rat diet given *ad libitum*. The three groups were designated normal, iron-loaded and iron-depleted and will be referred to as such throughout this paper. Before mating the animals of the different groups were treated as follows:

Normal; these were given no treatment.

Iron-loaded; these were given a 1% solution of ferrous ammonium sulphate *ad libitum* as their drinking water from the time they were weaned until they were mated.

Iron-depleted; in the rats of this group, during the last 2 weeks before mating, a measured quantity of blood was taken from the tail on six different occasions. On the average a total of 4·5 mg (range 3·4–5·9 mg) of iron in the form of haemoglobin was removed from each rat.

The day of insemination was determined by daily examination of vaginal smears for spermatozoa. Six rats from each group were killed on the 13th, 17th and 20th days respectively after observing sperm (here called the 13th, 17th and 20th day of pregnancy) and on the 1st day after parturition. Six rats from each group were allowed to suckle their young and it was planned to kill them on the 20th day of lactation. However, the whole of the litters of one of the normal rats, two of the iron-loaded and three of the iron-depleted rats, died during the first few days after birth. As a result the data shown for the 20th day of lactation are derived from 5, 4, and 3 lactating rats and their litters for the normal, iron-loaded and iron-depleted groups, respectively. The six non-pregnant rats of the iron-loaded and iron-depleted groups were killed 17 days after withholding the ferrous ammonium sulphate solution or 17 days after the last haemorrhage, respectively.

Collection of samples and analytical methods. The rat was anaesthetized with ether. The abdomen and chest were opened and blood was withdrawn from the heart with a syringe and needle previously moistened with heparin solution. The liver and spleen were removed and weighed. In the pregnant rat the uterus was opened, the foetal membranes divided and the umbilical vessels severed with a diathermy needle to prevent haemorrhage. The foetuses and the combined placentae plus foetal membranes were removed and weighed. All the foetuses and placentae plus membranes from any one litter were weighed and stored together as a pool. The new-born and 20-day-old rats were killed with ether, weighed and stored intact. The maternal livers and spleens were removed and weighed and samples were cut, weighed and stored. The maternal-tissue samples, the foetuses and new-born and the placentae were stored at $-16°$ C until analysed. A sample was taken from the freshly drawn blood and diluted for haemoglobin estimation by the method of Walsh, Arnold, Lancaster, Coote & Cotter (1953), and the remainder centrifuged at 2000 *g* for 15 min to obtain plasma. Plasma iron and total iron-binding capacity (TIBC) estimations were performed immediately by the method of Morgan & Carter (1960). Table 1 shows the different measurements performed on the various tissues:

TABLE 1

Tissue	Measurement
Mother	
Liver and spleen	Total storage (total non-haem) iron
	Ferritin (water-soluble non-haem) iron
Placenta plus membranes	Total non-haem iron
	Water-soluble non-haem iron
Offspring	
13-day foetus	Total iron
17- and 20-day foetus and new-born	Total iron
	Total non-haem iron
	Water-soluble non-haem iron
20-day suckling	Total iron
	Total non-haem iron

The tissue iron estimations were performed in duplicate on samples of homogenates of the tissues or whole animals. The liver and spleen samples from adult rats and the 13-day foetuses were homogenized in a wet grinder. The older foetuses and young rats and the placentae were homogenized in a Waring blendor. All the foetuses or young rats or all the placentae from any one litter were homogenized together. Total non-haemoglobin (non-haem) iron and water-soluble non-haem iron estimations were performed by the methods described by Kaldor (1954, 1958). For the total-body-iron estimations samples of the homogenized animals were wet-ashed with sulphuric, perchloric and nitric acids. Iron was measured in the digest absorptiometrically with orthophenanthroline reagent, as described by Kaldor (1958).

RESULTS

Pregnant and lactating rats

Haemoglobin, plasma iron and TIBC. The changes in blood haemoglobin, plasma iron and TIBC values are shown in Fig. 1. With one exception no statistically significant differences were found between the mean values for the three groups, and the results for the three groups have therefore

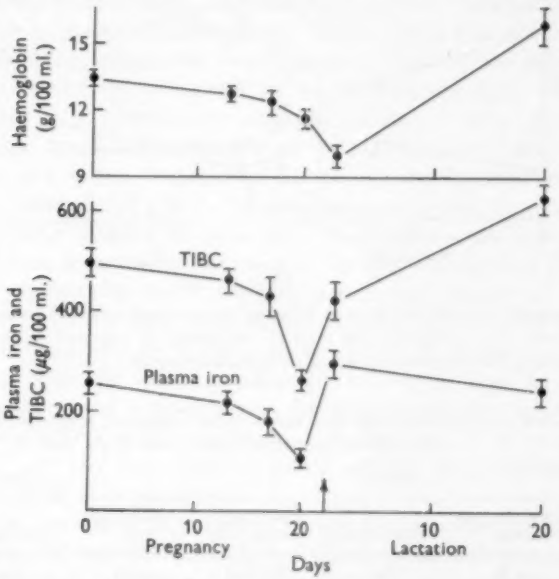

Fig. 1. Changes in blood haemoglobin concentration, plasma iron concentration and total iron-binding capacity (TIBC) during pregnancy and lactation in the rat. The time of parturition is indicated by the arrow. The vertical lines represent ± one standard error of the mean.

not been represented separately. For similar reasons the results from the three groups have been shown as single curves in Figs. 3 and 4. On the first day after parturition the TIBC was significantly greater in the iron-depleted than in the iron-loaded rats ($P < 0{\cdot}05$).

The blood haemoglobin concentration fell during pregnancy with a minimum value on the first day after parturition and had returned to the non-pregnant value on the 20th day of lactation. The plasma-iron concentration, however, behaved differently, a minimum value being reached on the 20th day of pregnancy, while on the 1st and 20th days after parturition values were the same as in non-pregnant animals. Changes in TIBC

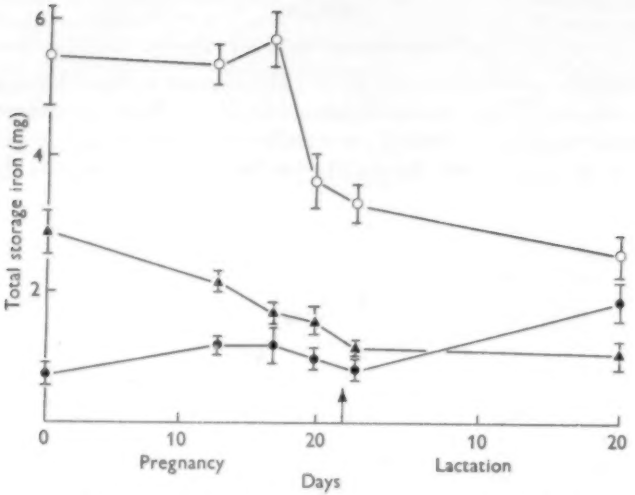

Fig. 2. Changes in the total storage iron found in the liver and spleen of maternal rats during pregnancy and lactation. The time of parturition is indicated by the arrow. ▲ Normal group; ○ iron-loaded group; ● iron-depleted group.

TABLE 2. Liver and spleen storage iron values in non-pregnant, pregnant and lactating rats (mean values ± standard error of the mean)

Day of pregnancy or lactation		Liver: Total storage iron concentration (μg/g)	Liver: Total storage iron content (mg)	Liver: Percentage storage iron in ferritin fraction	Spleen: Total storage iron concentration (μg/g)	Spleen: Total storage iron content (mg)	Spleen: Percentage storage iron in ferritin fraction
Normal group							
Non-pregnant		351 ± 68·00	2·23 ± 0·216	90 ± 2·14	729 ± 37·91	0·47 ± 0·081	84 ± 2·18
Pregnant	13	208 ± 32·60	1·82 ± 0·127	91 ± 1·13	394 ± 27·38	0·31 ± 0·032	89 ± 2·05
	17	194 ± 25·07	1·53 ± 0·128	94 ± 4·25	234 ± 39·92	0·18 ± 0·031	92 ± 2·24
	20	136 ± 25·19	1·29 ± 0·173	87 ± 3·08	251 ± 60·75	0·19 ± 0·039	85 ± 3·67
Lactating	1	93 ± 8·92	0·97 ± 0·098	94 ± 4·19	248 ± 56·98	0·16 ± 0·013	85 ± 1·60
	20	81 ± 18·88	0·93 ± 0·197	93 ± 2·04	101 ± 31·78	0·06 ± 0·018	81 ± 1·81
Iron-loaded group							
Non-pregnant		640 ± 98·15	4·69 ± 0·201	92 ± 3·96	1100 ± 93·51	0·79 ± 0·206	82 ± 3·17
Pregnant	13	591 ± 34·96	4·67 ± 0·274	95 ± 1·04	841 ± 156·9	0·68 ± 0·075	86 ± 2·41
	17	560 ± 60·15	5·12 ± 0·338	86 ± 2·13	697 ± 118·9	0·61 ± 0·095	83 ± 3·13
	20	399 ± 68·28	3·20 ± 0·317	92 ± 3·54	633 ± 120·3	0·39 ± 0·045	89 ± 3·28
Lactating	1	273 ± 31·20	2·99 ± 0·309	90 ± 4·22	671 ± 59·51	0·45 ± 0·024	84 ± 1·64
	20	281 ± 56·50	2·29 ± 0·226	89 ± 2·25	395 ± 61·20	0·17 ± 0·036	77 ± 2·04
Iron-depleted group							
Non-pregnant		109 ± 9·97	0·67 ± 0·132	87 ± 4·05	104 ± 26·15	0·06 ± 0·039	83 ± 2·99
Pregnant	13	153 ± 15·94	1·08 ± 0·079	92 ± 203	101 ± 9·60	0·07 ± 0·031	84 ± 3·69
	17	105 ± 24·23	1·07 ± 0·203	88 ± 1·20	104 ± 27·95	0·08 ± 0·056	83 ± 3·32
	20	99 ± 11·21	0·88 ± 0·101	91 ± 1·69	86 ± 11·04	0·06 ± 0·020	84 ± 2·35
Lactating	1	72 ± 12·95	0·73 ± 0·132	94 ± 2·98	77 ± 11·81	0·05 ± 0·025	95 ± 2·54
	20	156 ± 15·22	1·66 ± 0·289	96 ± 4·78	226 ± 16·82	0·11 ± 0·124	84 ± 2·18

were similar to those in plasma iron during pregnancy, but there was a further increase during lactation so that on the 20th day after parturition the TIBC was significantly greater than the non-pregnant value ($P < 0{\cdot}001$).

Storage iron in liver and spleen. In Fig. 2 are shown the changes found in the sum of the total storage iron contents of liver and spleen during pregnancy and lactation. In the latter part of pregnancy the average values decreased in all groups, the magnitude of decrease varying inversely with the level of storage iron. During lactation there was a further decrease in this value in the iron-loaded group. Although the iron stores of normal and iron-depleted groups were much the same at the end of pregnancy the two groups differed in one respect, i.e. during lactation there was a significant increase ($P < 0{\cdot}02$) in iron stores in the iron-depleted group but no change in the normal. The changes are set out in more detail in Table 1. Both liver and spleen contributed to the changes in total storage iron during pregnancy in all groups. During lactation, however, stores in liver and spleen were affected differently in the three groups. In the normal group the liver iron content showed no further change and there was a small but statistically significant decrease in the content of the spleen ($P < 0{\cdot}001$), with no significant change when stores in liver and spleen were added together. In the iron-loaded group there was no change in liver storage iron, the values remaining much higher than in the normal group, but there was a considerable decrease in splenic storage iron and thus a statistically significant decrease in the sum for liver and spleen ($P < 0{\cdot}05$). In the iron-depleted group storage iron increased during lactation in both liver and spleen.

In general, corresponding changes in the concentration of storage iron accompanied changes in content. It will be noted, however, that there was no significant alteration at any stage in the percentage of total storage iron found in the ferritin fraction.

Placenta

The changes in placental non-haem iron are shown in Fig. 3. At no time during pregnancy was a significant difference found between the mean values for any of the three groups. The placental non-haem iron content increased between the 13th and 17th days of pregnancy but no further change occurred between the 17th and 20th days. The non-haem iron concentration similarly increased between the 13th and 17th days but decreased significantly between the 17th and 20th days ($P < 0{\cdot}001$). The distribution of placental non-haem iron between the water-soluble and water-insoluble fractions remained constant during pregnancy with a mean value of 76·3% (S.E. $\pm$ 1·911) of the non-haem iron in the water-soluble fraction.

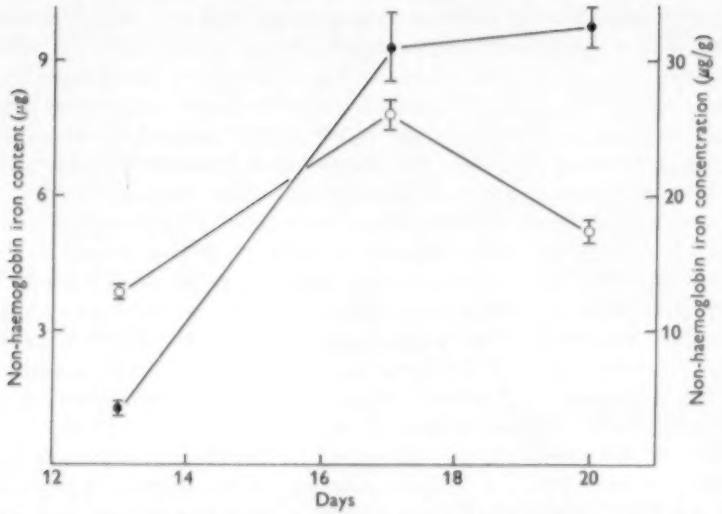

Fig. 3. Changes in the non-haemoglobin iron content (●——●) and concentration (○——○) in the placenta during pregnancy in the rat.

Foetal and suckling rats

The results are summarized in Fig. 4. There were no significant differences in the iron values for foetal and suckling rats between control, iron-loaded and iron-depleted groups. The total-body-iron content of the foetuses increased most rapidly between the 17th day of pregnancy and parturition. However, between the 13th and 20th days of pregnancy foetal iron increased more rapidly than foetal weight so that total-body-iron concentration increased, while between the 20th day and parturition the changes were proportional, so that no further change in concentration occurred. The total body non-haem iron concentration remained unchanged between the 17th day of pregnancy and parturition. There was also no change during this period in the proportion of non-haem iron which was water-soluble ($82{\cdot}3 \pm 1{\cdot}72\%$).

During suckling the total-body-iron content increased almost three times. However, body weight increased more rapidly than iron content so that total-body-iron concentration fell. The total body non-haem concentration also decreased during suckling.

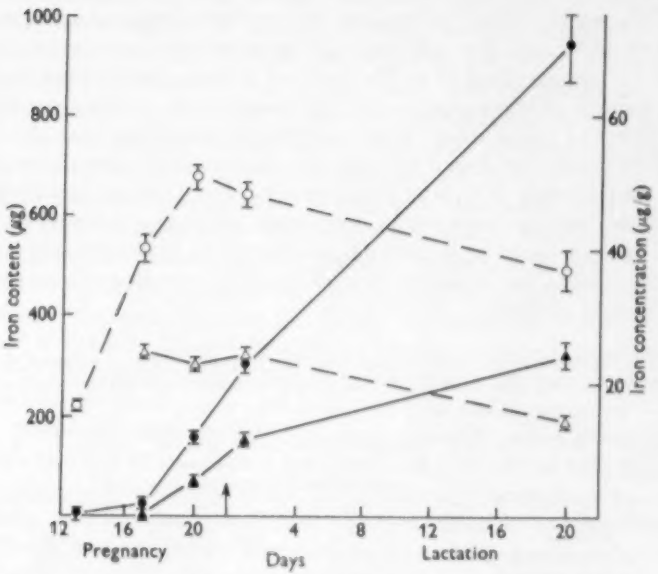

Fig. 4. Changes in total body iron content (●——●) and concentration (○——○) and in total body non-haemoglobin iron content (▲——▲) and concentration (△——△) during pregnancy and lactation in the rat. The time of parturition is indicated by the arrow.

DISCUSSION

An important purpose of the present investigation was to study the effects of experimentally produced alterations in the maternal iron stores on plasma iron and TIBC during pregnancy and on the transfer of iron to the offspring. The iron stores were altered before and not during pregnancy so that the conditions in the three groups during pregnancy should be as similar as possible, except for the difference in the size of maternal iron stores. Haemorrhage during pregnancy, as used by Nylander (1953), causes marked iron depletion in the mothers but it also produces anaemia and a considerably enhanced rate of erythropoiesis. Although in the present investigation the rats were probably mildly anaemic at the time of mating, they had recovered from this by the 13th day of pregnancy (Fig. 1) and the rate of erythropoiesis was by then probably similar in these animals to that in the other two groups. Alterations in the maternal iron stores of the degree produced in this experiment were not associated with any changes in maternal plasma iron and TIBC levels or in the placental or foetal iron values. It has been shown that the source of iron

transferred to the foetus is maternal plasma iron (Vosburgh & Flexner, 1950). The supply of iron to the placenta and foetus then must depend on the concentrations of iron and iron-binding protein in the maternal plasma and on the uterine blood flow. The first two of these factors were found to be the same in all three groups, and it is reasonable to assume that uterine blood flow was also similar. It is therefore not surprising that no significant differences were found between the placental and foetal iron values of the three groups. Nylander (1953) showed that if the rat was subjected to repeated haemorrhages during pregnancy the placental transfer of iron to the foetuses could be considerably reduced. In his experiments, however, the plasma iron concentration of the bled animals was considerably lower than in controls.

TABLE 3. Comparison of the loss of storage iron from liver plus spleen of maternal rats with the gain in total body iron of their offspring during pregnancy and during lactation (mean values)

	Storage-iron loss from pregnant or lactating rat (mg)	Total iron gain by litter of foetuses or suckling rats (mg)	Difference (mg)
Pregnancy			
Normal	2·8	3·4	0·6
Iron-loaded	3·2	3·1	−0·1
Iron-depleted	−0·1	2·2	2·3
Lactation			
Normal	0·1	3·4	3·3
Iron-loaded	0·7	3·2	2·5
Iron-depleted	−1·0	3·5	4·5

The iron transferred to the foetuses via the placenta or to the suckling rat via the milk could be derived from the maternal haemoglobin mass, the maternal iron stores or from iron absorbed from the diet. The total haemoglobin mass does not decrease during pregnancy or lactation in the rat (Bond, 1948, 1958; Brown & Pike, 1960). In the present work no differences in haemoglobin concentration were found in the different groups, so that maternal haemoglobin was not a likely source of the iron transferred to the foetuses or suckling animals. The results of the present investigation indicate that the amount of iron mobilized from the maternal stores for the needs of the developing conceptus depends largely on the amount present in those stores. When stores were depleted before mating and the pregnant rats were maintained on a diet adequate in iron, sufficient iron could be obtained, presumably from this diet, to meet the foetal demands. In Table 3 are shown the mean values in the three different groups for the changes in the sum of the storage iron contents of the liver and spleen during pregnancy and during lactation and the corresponding changes in iron content per litter of foetal and suckling rats. The difference

between the maternal change and changes in the foetal or suckling rat indicates very roughly the amount of iron absorbed during the period involved in excess of the amount normally absorbed. It is apparent that an increase in iron absorption occurred during both pregnancy and lactation and that during pregnancy at least the degree of this increase appears to be related inversely to the amount of storage iron present in the pregnant animal before mating. An increase in the efficiency of iron absorption during pregnancy has been demonstrated in man with radioactive iron (Balfour, Hahn, Bale, Pommerenke & Whipple, 1942; Hahn, Carothers, Darby, Martin, Sheppard, Cannon, Beam, Densen, Peterson & McClellan, 1951). The importance in the rat of dietary iron as a source of iron for transfer to the foetuses has been shown by Alt (1938). He found that if the pregnant rat was fed on a milk diet low in iron from the time of mating the new-born rats contained approximately half as much iron as the new-born rats of those fed on the stock diet or the milk diet with added iron.

The decrease in liver storage iron concentration found in the pregnant and lactating rats of the control group was very similar to that reported by Widdowson & McCance (1948). The reason why the storage iron concentration does not decrease during lactation, even though considerable quantities of iron are being transferred to the suckling young in the milk, is uncertain. It is probable that in the rats used in the present experiment, and also possibly in Widdowson & McCance's animals, the level of liver storage iron during lactation (about 80 μg/g liver tissue) represents almost a minimum value, beyond which the so-called storage iron cannot be reduced when the diet contains adequate iron. Chronic haemorrhage produced experimentally in non-pregnant female rats fed on a diet adequate in iron was found to reduce the liver storage iron concentration to 70–80 μg/g but no further, while haemorrhage in rats fed on an iron-deficient diet did further lower liver storage iron (unpublished observations). The results of the present experiment show that a reduction in storage iron concentration occurs in the spleen as well as in the liver but the relative proportion of iron present in the two storage forms remains unchanged. It appears therefore that in both organs the storage iron is released equally readily from both the ferritin and haemosiderin fractions (Table 2).

The foetal total and non-haem body iron contents found on the 17th and 20th days of pregnancy were similar to those reported by Nylander (1953). The mean total iron contents of the new-born rats were between the value of about 0·25 mg reported by Alt (1938) and Lintzel *et al.* (1944) and the value of 0·34 mg reported by McCance & Widdowson (1951). The finding that approximately 50% of the iron content of the new-born rat was present as non-haem compounds agrees well with the results of Alt (1938)

and Lintzel *et al.* (1944). Much of this non-haem iron must represent storage iron which can be utilized for the formation of the essential haem compounds during early life, when rapid growth requires the formation of a considerable amount of haem compounds and when the supply of dietary iron in the milk may be limited. During lactation the concentration of iron in the suckling rats decreased (Fig. 4). These results are similar to those reported by Smythe & Miller (1929), Huggett & Widdas (1950) and McCance & Widdowson (1951). It is apparent, then, that a considerable quantity of iron is transferred to the suckling rat in the milk but not in sufficient amounts to maintain the body iron concentration at the level found at birth. Rat milk is richer in iron than the milk of many other species (Cox & Mueller, 1937).

The changes found in the placental non-haem iron are in good agreement with those reported by Nylander (1953). The concentration of non-haem iron in the placenta diminished during the last few days of pregnancy, at which time the transfer of iron to the foetus was occurring at a maximal rate. The proportion of placental non-haem iron found in the present work in the water-soluble and water-insoluble fractions remained constant during pregnancy. Evidence obtained with immunochemical and radiotracer techniques has shown that in the liver these two fractions represent ferritin and haemosiderin iron (Gabrio, Shoden & Finch, 1953). Nylander (1953) demonstrated the presence of ferritin and of granules with the histochemical properties of haemosiderin in the rat placenta. It is, then, quite probable that the two non-haem iron fractions estimated for the placentae represent ferritin iron and haemosiderin iron. The constancy of their relationship to one another in the placenta is a finding similar to that made for the maternal livers and spleens. That placental ferritin may play an important part in the transfer of iron from mother to foetus has been suggested by Wohler (1955) and by Heilmeyer (1958). It is not known whether haemosiderin also plays a part in this.

A fall in the blood haemoglobin concentration of the pregnant rat has been frequently reported (Scott, 1928; Sure, Kik & Walker, 1929; Mitchell & Miller, 1931; Van Donk, Feldman & Steenbock, 1934). This 'anaemia' is due to an increase in plasma volume, not to a decrease in total circulating red-cell mass (Bond, 1948). Van Donk *et al.* (1934) found that administration of supplemental dietary iron to the pregnant rat did not prevent the fall of blood haemoglobin concentration and they concluded that the 'anaemia' was not due to iron deficiency. The present work confirms this and shows that the changes of haemoglobin level which occur during pregnancy are relatively constant despite considerable variation in maternal iron stores.

A fall in plasma iron concentration has been frequently reported in the

pregnant human being (Laurell, 1947; Lundström, 1950) and Nylander (1953) found a similar change in the pregnant rat. In the present work a marked fall in plasma iron occurred during the last few days of pregnancy (Fig. 2), suggesting that at this time iron was being transferred most rapidly across the placentae. The decrease in plasma iron was of the same degree whether or not the maternal iron stores contained a considerable amount of iron (iron-loaded group) or little iron (iron-depleted and normal groups). This indicates that the decrease in plasma iron concentration was probably due, not to depletion of the iron stores, but to the rapid passage of iron from maternal plasma through the placentae to the foetuses. The sudden separation of the placentae at parturition would bring this process to an abrupt end and could cause the increase in plasma iron levels. A small increase in plasma iron concentration after parturition has been reported for man (Lundström, 1950).

The plasma TIBC increases greatly during pregnancy in man (Laurell, 1947; Fay, Cartwright & Wintrobe, 1949). The marked decrease in TIBC during pregnancy in the rat is a puzzling feature and is difficult to explain in terms of our present knowledge of the processes involved. It could be due to a decrease in the rate of synthesis and/or an increase in the rate of destruction of transferrin, to an increase in the plasma volume without a corresponding increase in the total amount of circulating transferrin or possibly to the passage of transferrin through the placenta, sequestration by the placenta or destruction by the placenta. If alterations in the rate of transferrin formation or destruction within the pregnant rat were the cause of the decrease in TIBC it might be expected that these processes would revert to normal relatively slowly after parturition. The rapidity with which the TIBC increased after parturition would then be unexpected and suggests that some other explanation should be sought. An increase in plasma volume does occur in pregnancy in the rat (Bond, 1948). That an increase in plasma volume need not cause a reduction in TIBC is indicated by the fact that in man, where the plasma volume increases considerably during pregnancy (Diekman & Wegner, 1934; Roscoe & Donaldson, 1946), an increase in TIBC also occurs. Furthermore, changes in plasma volume cannot explain why the blood haemoglobin concentration fell and the TIBC rose immediately after parturition, since any blood loss or redistribution of body water would have affected haemoglobin and transferrin concentrations in a similar way. The last explanation for the fall in TIBC, that of placental passage, sequestration or destruction of transferrin, must receive full consideration since the loss of the placentae at parturition was followed by an abrupt rise in TIBC. No direct experimental evidence is available on these possibilities, although Dancis & Shafran (1958) have shown that in the guinea-pig towards the end of pregnancy β_1-globulin,

the plasma protein fraction in which transferrin is found, as well as other plasma protein fractions can pass through the placenta.

SUMMARY

1. The changes in maternal blood haemoglobin, plasma iron and total iron binding capacity (TIBC) and iron stores and in the accumulation of iron by the placentae and offspring, were measured in three groups of rats. The animals of one group received no treatment before mating (normal group) those of another group had their iron stores increased by feeding extra iron before mating (iron-loaded group) and the rats of the third group had their iron stores depleted by haemorrhage before mating (iron-depleted group).

2. The maternal blood haemoglobin concentration fell during pregnancy and immediately after parturition and rose again during lactation. The changes were of the same degree in the three groups.

3. The plasma iron concentration and TIBC both fell in all groups during the last few days of pregnancy and both rose immediately after parturition. During lactation the plasma iron remained at the non-pregnant level while the TIBC rose to values above that level.

4. The maternal iron stores (liver plus spleen) fell during pregnancy in both the iron-loaded and normal groups, the rate of fall being greater in the iron-loaded group. In the iron-depleted group the iron stores after parturition were the same as in non-pregnant controls. During lactation the iron stores decreased further in the iron-loaded group, remained unchanged in the normal group and increased in the iron-depleted group. In general, these changes occurred in a similar manner in both liver and spleen and without any significant alteration in the proportions of iron in the two fractions, ferritin and haemosiderin.

5. The non-haemoglobin iron values for the placenta were the same in the three groups. The concentration fell during the last few days of pregnancy at which time iron was being transferred most rapidly to the foetus. No alteration occurred in the relative distribution of the placental non-haemoglobin iron between its water-soluble and water-insoluble fractions.

6. The foetuses accumulated iron most rapidly during the last four days of pregnancy. During lactation the total body iron of the sucklings increased two to three times. No difference was found between the three groups in the amount or concentration of iron found in the foetuses or sucklings.

7. It is concluded that in the rat on a normal diet during pregnancy considerable alterations in the size of the iron stores induced experimentally before mating result in no changes in maternal haemoglobin, plasma iron

and TIBC values or in the transfer of iron to the offspring by the placenta or milk.

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